STUDIES ON THE NATURE OF ANTIBODY PRODUCTION DURING THE *IN VITRO* CULTURE OF LYMPHOID TISSUES[1]

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(*Accepted for publication 1st July, 1960.*)

SUMMARY.

A simple and reproducible technique for the study of the production of antibodies by lymphoid tissues during *in vitro* culture has been described.

Cellular and extra-cellular fluid titres were estimated by Boyden's tanned cell technique, and a sharp rise in the fluid antibody titre (up to 300-fold) was observed during the first 18 to 24 hours of culture, while the cellular titre remained at a low constant level. The results of this study suggest that antibody-producing cells store little or no classical antibody.

The presence of chloramphenicol in the culture medium inhibited antibody production, while production was not specifically inhibited by ribonuclease. In addition, antibody production was inhibited by the absence of Ca++ from the culture medium and this effect was reversed when Ca++ was restored in physiological concentration.

Attempts to stimulate antigenically spleen and popliteal node tissues during *in vitro* cultures were not successful.

INTRODUCTION.

During recent years many workers have focussed their attention upon the production of antibody by lymphoid tissues cultured *in vitro*. These studies have been concerned with the production of antibody by tissue slices of rabbit spleen, and mesenteric and popliteal node maintained by stationary or roller tube culture. The optimal conditions for the continued production of antibody *in vitro*, following antigenic stimulation of the mesenchymal tissues *in vivo*, have been defined; and attempts have been made to demonstrate that the increase of specific antibody titre, which is invariably observed during culture of the tissues, is due to nett protein synthesis from free amino acids rather than release of mature antibody, or activation of a precursor which may have been pre-formed *in vivo* (Askonas and White, 1956; Stavitsky, 1955; Wolf and Stavitsky, 1956; Stavitsky and Wolf, 1958). While Harris and Harris (1955) have demonstrated that the induction phase of antibody production can be carried out by incubation of lymphoid cells with antigens *in vitro*, and transfer of the stimulated cells

[1] This work was assisted by a grant from the National Health and Medical Research Council, Canberra.

to X-irradiated animal hosts, the many attempts which have been made to initiate and to produce antibody by culture of lymphoid tissues in an *in vitro* environment, have not yielded convincing results, although Stevens and McKenna (1957) have described some studies which suggest that both induction and synthesis of antibody might be achieved under the conditions imposed by their *in vitro* system and technique.

Our studies describe a simple technique for the investigation of the synthesis of antibody *in vitro*, definition of some of the properties of the system, and attempts to confirm the work of Stevens and McKenna. We have also investigated the effects of chloramphenicol and ribonuclease on the production of antibody during the *in vitro* culture of lymphoid tissues.

Materials and Methods.

Animals. The animals used in the present experiments were crossbred rabbits of about 2½ kilograms in weight. No attention was paid to the sex of the animals.

Antigens and control antisera. Bovine serum albumin (BSA) and gamma globulin (BGG) were alum precipitated by the method described by McKenna and Stevens (1956). Alum precipitated diphtheria toxoid (50 Lf/ml.) was obtained from the Commonwealth Serum Laboratories, Melbourne. The hyper-immune antisera used to control the specificity of the antibody titrations were obtained by bi-weekly stimulation of three rabbits with the antigens over a period of four weeks. Twenty Lf units of diphtheria toxoid and 40 mg. of alum precipitated BSA and BGG were given intravenously at each injection. The rabbits were bled from the marginal ear veins two weeks after the final injection and the hyper-immune sera were stored in 1 ml. ampoules at — 20° C.

Titration of antibody. Antibodies were titrated by the method of Fisher's modification of the Boyden tanned-cell technique (Fisher, 1952). Fisher established that a good correlation existed between the haemagglutinating titre and the antitoxin titre of sera as determined by the Schick intradermal test. The tanned-cell technique was both sensitive and reproducible in his hands. The hyper-immune sera used in our studies usually titrated out to a dilution of 1/30,000 to 1/60,000 in the diphtheria system, $1/2 \times 10^5$ in the BGG system, and $1/5 \times 10^5$ in the BSA system.

Tissue culture fluids and cellular antibody extracted into physiological saline were absorbed first with normal and then with unsensitised tanned sheep's red blood cells as non-specific agglutination of tanned cells was frequently observed when these preparations were absorbed with normal sheep's erythrocytes alone.

The method of tissue culture. Spleens and popliteal nodes were removed with aseptic precautions from antigenically stimulated animals into Earle's saline. The tissues were then finely diced in the saline with sharp surgical scissors and gently pipetted for five to ten minutes to release single cells and small cell clumps. The tissue preparations thus obtained were usually washed twice in Earle's saline by centrifugation at 700 r.p.m. in an angle centrifuge for five minutes and the sedimented tissue and cells were resuspended by gentle pipetting. After the final washing, the tissues were suspended in the requisite volume of culture medium (see Experimental) and dispensed in 1 ml. volumes into roller-tubes. The cultures were incubated at 37° C. and rotated at 40 rev. per hour.

The extraction of antibody from tissues. When fluid and cellular antibody levels of culture specimens were determined separately, the supernatant culture fluids were carefully pipetted from the cultured tissues and centrifuged at 4,000 r.p.m. for 15 minutes to remove contaminating tissue or cell debris. Antibody was extracted from the cultured tissues either

by grinding the frozen tissues in a mortar or by homogenisation with a Potter-Elvehjem pestle and mortar. These treatments did not reduce the titre of a hyper-immune serum in the presence of normal rabbit spleen. After extraction of the cellular antibody into saline, the disrupted cells were removed by centrifugation.

EXPERIMENTAL.

We have observed, in agreement with Stavitsky (1955), that a time lag of at least three to four weeks must be allowed between primary and secondary *in vivo* stimulation of spleen and popliteal nodes so that maximum production of antibody might occur when these tissues are subsequently cultured *in vitro*. Spleens from animals injected intravenously, and popliteal nodes from animals injected with antigen into the hind foot-pads, were removed two or three days after secondary stimulation. Stavitsky and Wolf (1958) have recently shown that the maximum production of antibody occurs *in vitro* when lymphoid tissues are set up in culture three days after secondary *in vivo* stimulation.

The tissue culture medium.

The effect of the addition of autologous and of homologous normal rabbit serum. Two stock rabbits (1087 and 1088) were bled from the marginal ear veins before antigenic stimulation to obtain autologous and homologous normal sera. Rabbit 1087 was then injected with 20 mg. of alum-precipitated BSA into the left hind foot-pad and rabbit 1088 was injected with 20 Lf units of diphtheria toxoid into the right hind foot-pad. Three weeks later a secondary immunological response was induced by the injection of the antigens by the same routes and in the same dosage. Two days after the second course of injections the animals were sacrificed and half of the spleen and half of the homolaterally stimulated node from each animal was diced in 6 ml. of Earle's saline. The tissue preparations were then divided into three aliquots with a 0·5 ml. pipette. The remaining halves of the antigenically stimulated tissues were used in an experiment designed to show the effect of antigenic stimulation *in vitro* (see—Attempts to initiate antibody production *in vitro*). Five millilitres of Earle's saline and 1 ml. of autologous serum were added to the second aliquots, and 4 ml. of Earle's saline and 1 ml. of homologous serum were added to each of the third aliquots. Each aliquot was then dispensed in 1 ml. volumes into six roller-tubes and three tubes were randomly selected from each group and immediately frozen in a dry-ice and alcohol bath and stored at $-20°$ C. The remaining tubes were incubated at 37° C. for 48 hours.

The fluid and cellular contents of the three tubes selected from each group at 0 and 48 hours were pooled, and the cellular antibody extracted into their tissue culture fluids. Thus, both fluid and cellular antibody were titrated in the one culture specimen. The results of this experiment are given in Table 1.

The results show that higher antibody titres were obtained when spleen and homolaterally stimulated popliteal nodes were cultured in the presence of

TABLE 1.

The effect of the addition of autologous and homologous sera to the tissue culture medium during the culture of spleen and popliteal node.

	Hours after setting tissues up in culture			
	0 hours		48 hours	
	BSA sens. cells*	Dip. toxin sens. cells	BSA sens. cells	Dip. toxin sens. cells
Rabbit 1087—injected with BSA into left hind foot-pad				
Spleen				
in Earle's saline	512	<2	768	<2
in E.S.+autol. serum	384	<2	1536	<2
in E.S.+homol. serum	384	<2	1536	<2
Left Popliteal Node				
in Earle's saline	256	<2	1536	<2
in E.S.+autol. serum	384	<2	3072	<2
in E.S.+homol. serum	384	<2	3072	<2
Rabbit 1088—injected with Dip. toxoid into right hind foot-pad				
Spleen				
in Earle's saline	<8	96	<2	384
in E.S.+autol. serum	3	192	<2	768
in E.S.+homol. serum	3	96	<2	1024
Right Popliteal Node				
In Earle's saline	<2	192	<2	384
in E.S.+autol. serum	3	192	<2	1536
in E.S.+homol. serum	<2	384	4	1536

* Tanned cells sensitised by BSA.

serum. The results did not show, however, that autologous serum was a more effective nutrient or afforded better protection for antibody producing tissues in culture than homologous serum.

The high antibody titres which were observed in the 0 hour specimens can probably be attributed to contaminating pre-formed antibody, as no attempt was made to wash the tissues before culture. This hypothesis has been confirmed in subsequent experiments. Thus the rise in antibody titre which was observed during culture of the tissues, was probably much greater than the results of the present experiment suggest.

Antibody was more actively produced by the draining lymph node than by spleen when the antigen was injected into the foot-pad.

The experiment described above was repeated, but the tissues were washed once in Earle's saline before culture. The results given in Table 2 confirm that the production of antibody in tissue culture is increased in the presence of autologous and homologous serum. The low levels of antibody which were obtained in the 0 hour specimens after washing the tissues suggest that antibody-producing cells carry little or no antibody.

TABLE 2.

The effect of the addition of autologous and homologous sera to the tissue culture medium during culture of spleen and popliteal node, the tissues being washed prior to culture.

	Hours after setting tissues up in culture			
	0 hours		48 hours	
	BSA sens. cells	Dip. toxin sens. cells	BSA sens. cells	Dip. toxin sens. cells
Rabbit 1090—injected with BSA into left hind foot-pad.				
Spleen				
in Earle's saline	3	<1	24	<1
in E.S.+autol. serum	3	<1	32	<1
in E.S.+homol. serum	2	1	48	<1
Left Popliteal Node				
in Earle's saline	43	<1	128	<1
in E.S.+autol. serum	43	<1	384	<1
in E.S.+homol. serum	32	<1	384	<1
Rabbit 1091—injected with Dip. toxoid into right hind foot-pad.				
Spleen				
in Earle's saline	<1	<1	<1	10
in E.S.+autol. serum	<1	1·5	<1	48
in E.S.+homol. serum	<1	1	<1	48
Right Popliteal Node				
in Earle's saline	<1	4	<1	64
in E.S.+autol. serum	<1	4	1·5	128
in E.S.+homol. serum	<1	6	<1	192

The effect of various salt solutions and basal media on the production of antibody in tissue culture. Lymphoid tissues from antigenically stimulated rabbits were cultured in Trowell's glucose saline (Trowell, 1952), Eagle's basal medium (Eagle, 1955), the 199 medium of Morgan *et al.* (1950) and the T.A.C.P.I. medium used by Stevens and McKenna (1957), both with and without the addition of serum. The influence of culturing the tissues in an atmosphere of oxygen was also investigated. However, the amount of antibody produced by lymphoid tissues cultured in these media was on no occasion greater than when the tissues were cultured in a medium containing 25 p.c. rabbit serum in Earle's saline.

A typical result, which compares antibody production by spleen and popliteal and mesenteric nodes in Earle's saline and Trowell's glucose saline and in the presence and absence of oxygen, is shown in Table 3. Thirty per cent. autologous rabbit serum was added to the salt solutions.

The results given in Table 3 show that the production of antibody by the lymphoid tissues was not increased by culture in Trowell's glucose saline or by culture in an atmosphere of oxygen. It is apparent, however, that the mesen-

teric nodes were not as effectively stimulated by the foot-pad and intravenous injections of antigen as the popliteal nodes or spleen.

The "cellular" and extra-cellular fluid titres of antibody. During the course of the *in vitro* studies we observed that the titre of the 0 hours specimen could be drastically reduced by careful washing of the tissues before setting them up

TABLE 3.

Comparison of the production of antibody during the culture of lymphoid tissues in Earle's saline and Trowell's glucose saline and in the presence of oxygen.

Rabbit 1093— Injected with BSA into the hind foot-pads and intravenously	Hours after setting tissues up in culture		
	0 hours	6 hours	48 hours
Spleen			
in E.S.+autol. serum	8	32	384
in T.G.S.+autol. serum	6	32	256
in E.S.+autol. serum+O_2	—*	—	384
in T.G.S.+autol. serum+O_2	—	—	512
Popliteal Nodes			
in E.S.+autol. serum	8	48	256
in T.G.S.+autol. serum	4	32	384
in E.S.+autol. serum+O_2	—	—	384
in T.G.S.+autol. serum+O_2	—	—	192
Mesenteric Nodes			
in E.S.+autol. serum	2	3	32
in T.G.S.+autol. serum	<2	<2	48
in E.S.+autol. serum+O_2	—	—	24
in T.G.S.+autol. serum+O_2	—	—	16

* Specimen not titrated.

TABLE 4.

The levels of "cellular" and extra-cellular fluid antibody during tissue culture of spleen and node.

Rabbit 1094— Injected with Dip. toxoid into the hind foot-pads and intravenously	Hours after setting the tissue up in culture			
	0 hours	6 hours	18 hours	60 hours
Spleen				
Fluid antibody level	16*	64	192	1024
"Cellular" level		16	16	24
Fluid antibody level	16	80	384	1024
"Cellular" level		16	16	24
Popliteal nodes				
Fluid antibody level	64	192	1024	1536
"Cellular" level		64	80	48
Fluid antibody level	64	128	384	2048
"Cellular" level		64	64	48

* Titrations were made on pools of 3 cultures selected at random. The tissues were cultured in a medium containing 25 p.c. normal rabbit serum in Earle's saline.

in culture. If "cellular" and fluid titres were then estimated separately, the fluid antibody titre invariably rose sharply during culture of the tissues while the "cellular" titres remained constant throughout the course of the experiment and, within the bounds of experimental error, at the low level of antibody titrated in the 0 hours specimens (see Table 4). The inference that the cells

TABLE 5.

The effect of ribonuclease on antibody synthesis in vitro.

		Hours after setting tissues up in culture				
		0	6	18	42	68
Rabbit 1098—injected with Dip. toxoid into the hind foot-pads and intravenously						
Spleen						
in E.S.+25 p.c. autol. serum		10	—	512	2048	2048
in E.S.+25 p.c.+0·05 mg. RNase/ml.		8	—	512	2048	2048
in E.S.+25 p.c.+0·50 mg. RNase/ml.		12	—	384	2048	2048
in E.S.+25 p.c.+5.00 mg. RNase/ml.		12	—	192	2048	2048
Popliteal node						
in E.S.+25 p.c. autol. serum		16	—	512	2048	2048
in E.S.+25 p.c.+0·05 mg. RNase/ml.		20	—	1024	2048	2048
in E.S.+25 p.c.+0·50 mg. RNase/ml.		32	—	768	2048	2048
in E.S.+25 p.c.+5·00 mg. RNase/ml.		48	—	384	2048	2048
Rabbit 1111—injected with Dip. toxoid into the hind foot-pads and intravenously						
Spleen						
in Ca-free E.S.+25 p.c. autol. serum		12	—	1024	4096	4096
in Ca-free E.S.+0·01 mg. RNase/ml.		12	—	1024	4096	4096
in Ca-free E.S.+0·10 mg. RNase/ml.		16	—	1024	4096	4096
in Ca-free E.S.+3·00 mg. RNase/ml.		16	—	1024	3072	4096
Popliteal node						
in Ca-free E.S.+25 p.c. autol. serum		16	—	768	2048	2048
in Ca-free E.S.+0·01 mg. RNase/ml.		16	—	768	2048	2048
in Ca-free E.S.+0·10 mg. RNase/ml.		16	—	768	2048	2048
in Ca-free E.S.+3·00 mg. RNase/ml.		16	—	768	2048	2048
Rabbit 1095—injected with BSA into the hind foot-pads and intravenously						
Spleen			*M.C.			
in E.S.+25 p.c. autol. serum	Fl.	8	16	32	640	
	C.		6	6	12	
in E.S.+25 p.c.+0·30 mg. RNase/ml.	Fl.	8	16	24	512	
	C.		6	6	6	
in E.S.+25 p.c.+3·00 mg. RNase/ml.	Fl.	8	16	16	256	
	C.		6	6	8	
Popliteal node						
in E.S.+25 p.c. autol. serum	Fl.	12	96	96	2048	
	C.		12	12	24	
in E.S.+25 p.c.+0·30 mg. RNase/ml.	Fl.	12	48	48	2048	
	C.		8	12	24	
in E.S.+25 p.c.+3·00 mg. RNase/ml.	Fl.	12	48	64	1024	
	C.		12	8	12	

* Medium changed.

which are actively engaged upon antibody synthesis, store little or no antibody, may be drawn from these observations.

The effect of ribonuclease on in vitro *antibody production.* Many workers have shown that protein synthesis is intimately associated with ribonucleic acids and that the synthesis of virus, bacterial, plant and animal proteins can be inhibited by ribonuclease (for review see Brachet, 1957). Following these studies, we have investigated the effect of ribonuclease on the synthesis of antibody by rabbit spleen and popliteal node in tissue culture. Thus, the lymphoid tissues were harvested from rabbit 1098, 48 hours after secondary antigenic stimulation with diphtheria toxoid. Aliquots of the tissues were cultured in a medium consisting of 25 p.c. autologous serum in Earle's saline and replicate cultures were set up in the presence of a range of concentrations of ribonuclease, and with ribonuclease omitted from the medium. The results of this experiment, which are given in Table 5, show that the ribonuclease did not inhibit the *in vitro* production of antibody.

Burnet, Lind and Perry (1957) studied the multiplication of influenza viruses in the de-embryonated egg in the presence of ribonuclease, and they showed that the inhibitory effect of the enzyme is enhanced if Ca^{++} is omitted from the saline medium. In an attempt to increase the inhibitory action of ribonuclease on antibody synthesis, and because we used the same preparation of ribonuclease which Burnet and his colleagues used to prevent virus production, we have followed their example and estimated the levels of antibody which occur during the culture of lymphoid tissues in a medium containing 25 p.c. Ca^{++}-free[2] autologous serum in Ca^{++}-free Earle's saline. However, production of antibody by the tissues was suppressed in the Ca^{++}-free medium *per se*. As a compromise, spleen and node tissues were cultured in a medium containing an intermediate concentration of Ca^{++} (25 p.c. whole autologous serum in Ca^{++}-free Earle's saline—Rabbit 1111). Again, inhibition of antibody production due to the enzymic action of ribonuclease was not detected (see Table 5).

Spleen and popliteal node tissues, removed 48 hours after secondary antigenic stimulation of rabbit 1095, were supported in Ca^{++}-free medium containing 0·3 and 3·0 mg. of ribonuclease per ml., for the first six hours of culture. Subsequent culture was then carried out in the complete medium (containing Ca^{++}) and in the continued presence of ribonuclease. This procedure was adopted in the hope that the absorption of ribonuclease might be facilitated by the temporary absence of Ca^{++}, while the protein synthesising mechanism might not be irreparably damaged. Specimens were harvested at 18 and 42 hours after setting the tissues up in culture and the results of this study are also included in Table 5. While the level of antibody produced by the culture of the lymphoid tissues in the presence of Ca^{++} increased 10- to 60-fold between

[2] The autologous sera were passed through an ion-exchange column (IR 120) to remove the Ca^{++}.

0 and 18 hours (rabbits 1098 and 1111), when the tissues were cultured in a Ca^{++}-free medium for the first six hours, the rise in titre which occurred between 0 and 6 hours was not continued between 6 and 18 hours and the nett increase in titre between 0 and 18 hours in these cultures was only two- to four-fold (rabbit 1095). However, the ability of the tissues to synthesise antibody was not irreversibly impaired by the temporary absence of Ca^{++}, as after the 12-hour lag between 6 and 18 hours, when no increase in the fluid antibody titre was detected, the levels of antibody in the culture fluids showed a 20- to 40-fold rise between 18 and 42 hours. This increase in titre was consistently greater than the rise produced during the same period by the tissues cultured in a medium containing Ca^{++} throughout the course of the experiment. As the cellular level of antibody remained at a low, constant level throughout the course of the *in vitro* culture, it is unlikely that the failure to detect a rise in titre in the fluid medium during the lag period was simply due to the inhibition of the release mechanism and consequent accumulation of intra-cellular antibody.

The inhibition of in vitro *antibody synthesis by chloramphenicol.*

The results of this experiment show that chloramphenicol, at a concentration of 5·0 mg. per ml., inhibited antibody synthesis by both spleen and node. Although the inhibitory concentration was high, our finding is substantially in

TABLE 6.

The inhibition of antibody production by chloramphenicol.

Rabbit 1112—injected with Dip. toxoid into the hind foot-pads and intravenously		Hours after setting the tissues up in culture			
		0	18	42	68
Spleen					
in E.S.+25 p.c. autol. serum	Fl.	4	96	384	192
	C.		6	11	6
in E.S.+25 p.c.+0·05 mg.* Ch/ml.	Fl.	4	96	192	192
	C.		6	5	3
in E.S.+25 p.c.+0·50 mg. Ch/ml.	Fl.	6	80	128	384
	C.		4	3	4
in E.S.+25 p.c.+5·00 mg. Ch/ml.	Fl.	4	5	5	16
	C.		2	2	2
Popliteal node					
in E.S.+25 p.c. autol. serum	Fl.	32	384	768	768
	C.		20	20	24
in E.S.+25 p.c.+0·05 mg. Ch/ml.	Fl.	32	384	768	1024
	C.		16	24	32
in E.S.+25 p.c.+0·50 mg. Ch/ml.	Fl.	40	256	512	512
	C.		20	16	32
in E.S.+25 p.c.+5·00 mg. Ch/ml.	Fl.	32	16	20	16
	C.		16	16	16

* Ch—Chloramphenicol.

agreement with studies on the action of chloramphenicol on protein synthesis in other systems. This result indicates that nett protein synthesis was involved in the production of antibody by the lymphoid tissues during *in vitro* culture.

Attempts to initiate antibody production in vitro.

Following the studies described by Stevens and McKenna (1957), we have made many attempts to initiate the synthesis of antibody in spleen and popliteal node culture. The methods described by Stevens and McKenna have been carefully followed in order that the conditions which they found favourable for the initiation and the continued production of antibody *in vitro* might be reproduced in this laboratory. However, as the *Salmonella* endotoxin which they used to stimulate spleen was not available, we substituted an *Haemophilus* influenzae (type B) polysaccharide endotoxin[3] with similar pharmacological properties, in our experiments. Thus far our attempts to confirm the work of Stevens and McKenna have not met with success.

In addition to the attempts to initiate antibody production *in vitro*, we have incubated spleen and node tissues from animals which had received a primary antigenic injection three weeks previously, with both the same antigen and an heterologous antigen, in order that a secondary homologous and heterologous antigenic stimulus might be provided *in vitro*. However, the cultured tissues failed to respond to this treatment.

At the present time we are not able to find an obvious technical fault which might explain our failure to induce antibody production in the *in vitro* system, although it is possible that the *Salmonella* endotoxin used by Stevens and McKenna may possess specific properties which are not shared by the toxin used in our experiments.

DISCUSSION.

The accuracy with which results were reproduced in replicate cultures was a striking feature of our studies. Under optimal conditions a 300-fold rise in the titre of antibody occurred in the extra-cellular medium, while 30- to 50-fold rises were regularly observed. Thus the method of *in vitro* culture of lymphoid tissues for the production of antibody described in the present communication was both effective and reproducible.

Generally the rise in titre which was observed in the extra-cellular medium was more rapid over the first 18 hours of culture than during subsequent culture of the lymphoid tissues. The fluid antibody was stable: it resisted standing at 37° C. for 48 hours and storage at – 20° C. for several months, and it is therefore likely that the decrease in the rate of antibody production during the course of culture was due to cessation of antibody formation rather than equilibration of

[3] Obtained through the courtesy of Dr. E. L. French, who obtained the preparation in 1948 from Dr. E. V. Keogh of the Commonwealth Serum Laboratories, Melbourne.

production and decay. However, when the tissues were cultured in a Ca^{++}-free medium for 6 hours and then cultured in the complete medium, the situation was reversed and a greater rise in the fluid titre was observed between 18 and 42 hours (see Table 5). This delayed occurrence of about the same maximum fluid titre suggests that lymphoid tissues can be maintained in a potentially functional state for periods much longer than 18 hours.

Alternatively, it might be contended that the results of the present studies reflect the release of antibody pre-formed *in vivo*, rather than production of antibody *in vitro* and that Ca^{++} is essential for the functioning of the release mechanism. However, pre-formed antibody was not released in sufficiently high titre by mechanical disruption of the stimulated lymphoid tissues and our studies show that only small amounts of classical antibody are stored intra-cellularly by comparison with the amounts of antibody which the tissues are able to produce during *in vitro* culture. In addition, chloramphenicol inhibited the *in vitro* production of antibody and this observation is consistent with the hypothesis that nett protein synthesis rather than the release of pre-formed antibody occurred *in vitro*.

Since the pioneer studies conducted by Caspersson (reviewed by Caspersson, 1950), an overwhelming volume of biochemical and cytochemical evidence has accumulated which clearly shows that ribonucleic acids play an essential rôle in protein synthesis. Many workers have, therefore, studied the inhibitory action of ribonuclease on protein synthesis in a variety of biochemical and biological systems (Brachet, 1955; Webster and Johnson, 1955; Castermann and Jeener, 1955; Ledoux and Baltus, 1954; Le Clerc, 1956; Burnet, Lind and Perry, 1957; and others). In an attempt to extend these studies, we have investigated the effect of ribonuclease on the *in vitro* production of antibody under the conditions imposed by the system and technique used in this laboratory. However, we were not able to demonstrate that the *in vitro* production of antibody was inhibited by ribonuclease.

Acknowledgments. We are much indebted to Sir Macfarlane Burnet, O.M., F.R.S., for his advice and guidance during the course of these investigations. In addition, we wish to acknowledge with gratitude, the valuable technical assistance given to us by Miss Rae Brady and by Miss Margaret McElroy.

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THE IMMUNOLOGICAL RESPONSE PRODUCED IN INBRED MICE FOLLOWING THE ISOLOGOUS AND HOMOLOGOUS TRANSFER OF ANTIGENICALLY STIMULATED SPLEEN CELLS[1]

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(*Accepted for publication 1st July, 1960.*)

SUMMARY.

Antigenically stimulated spleen cells from C3H and C57Bl mice have been transferred to isologous and homologous recipients and both the immediate appearance of antibody and the response to a challenge injection of antigen 11-12 weeks later have been studied.

Antibody can be detected as early as two days after isologous transfer of C57Bl cells, but none appears after homologous transfer. Isologous transfer of C3H cells gives much less evidence of early response showing either a delay up to six days before first appearance or failing completely.

Following late challenge mice that received isologous cells responded with typical secondary response in the great majority. No secondary type responses were obtained with homologous combinations.

INTRODUCTION.

During the present studies we have explored the differences in response produced by the transfer of antigenically stimulated lymphoid cells both isologously, and homologously, in two inbred strains of mice (C3H and C57Bl). Our studies include an investigation of the serum antibody levels in recipients immediately after transfer of spleen cells from secondarily stimulated donor mice and in the recipient animals following appropriate antigenic stimulation given three months after cell transfer. Osmotically lysed homogenates of the stimulated lymphoid cells were also transferred in a preliminary attempt to isolate a sub-cellular element which might induce the production of circulating antibodies similar to the factor described by Lawrence (1956) for the transfer of delayed hypersensitivity in man. A detailed account of the preliminary experiment has not been included in this report, as all attempts to isolate such a subcellular factor were unsuccessful. However, attention is drawn to this negative

[1] This work was assisted by a grant from the National Health and Medical Research Council, Canberra.

finding as this supports the contention that the responses observed in the recipient animals following whole cell transfer were due to the transferred cells and not due to stimulation of the lymphoid tissues of the recipients by native antigen.

The studies described in this communication show that all phases of the immune response can only be transferred in an isologous system and the implication of this finding is relevant both to the mechanism of antibody production and to the mechanism of the rejection of homotransplanted cells.

MATERIALS AND METHODS.

The design of the experiments.

A preliminary experiment was conducted to characterise the primary and the secondary serological responses in C3H and C57Bl strains of mice following the injection of diphtheria toxoid. Antigenic challenge was given three weeks after primary insult and 1·0 ml. of alum-precipitated diphtheria toxoid[2] (containing 8 Lf of precipitated toxin) was injected intraperitoneally into each mouse for both primary and secondary injections. When this procedure was followed the primary immunological response was clearly distinguishable from the secondary response in both strains of mice. The ability to produce and recognise a primary or a secondary response following antigenic stimulation was highly relevant to the design and to the interpretation of the results of subsequent experiments.

Cells obtained from pools of 12 spleens harvested from both strains of mice at 40 hours (experiment 2), six days (experiment 1) and seven days (experiment 3) were transferred both isologously and homologously into normal animals. The cells were carefully washed to avoid contamination either with extra-cellular native antigen or with antibody, and 35×10^6 cells in 1 ml. of Earle's saline were injected intraperitoneally into each recipient mouse. In addition, lysed-cell homogenates, obtained by successive freezing and thawing, were transferred both isologously and homologously into normal animals in experiments 1 and 3. Six animals were injected in each group.

The levels of serum antibody were titrated in the groups of animals of experiment 2 at intervals immediately following isologous and homologous whole-cell transfer, and in the groups of animals of experiment 3 immediately following both isologous and homologous whole-cell and homogenate transfers. No such titres were estimated in the sera of the groups of animals of experiment 1.

The recipient groups were bled 11 weeks after transfer in experiment 1 and 12 weeks after transfer in experiments 2 and 3, and all mice were then challenged intraperitoneally with diphtheria toxoid. Concurrently, groups of six untreated control animals were given primary injections of diphtheria toxoid. The levels of serum antibody in both treated and control groups of mice were titrated at frequent intervals following the antigenic injections. The general experimental procedure is represented graphically in Fig. 1.

Animals.

Adult (8-10-week-old) male C3H and C57Bl mice maintained by sibling matings were used throughout the present studies.

Preparation of spleen cells and lysed-cell homogenates for transfer.

Spleen cells. Spleens were removed with aseptic precaution from groups of 12 mice at the appropriate intervals following secondary antigenic stimulation (see above) and

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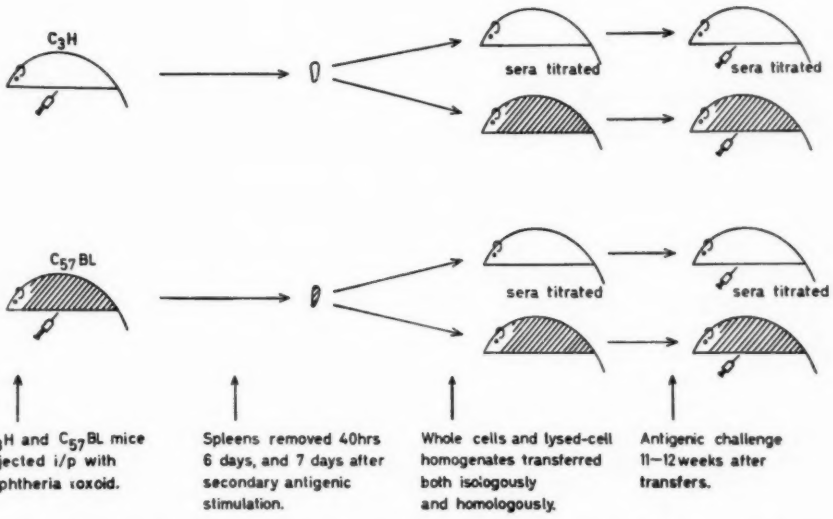
[2] Commonwealth Serum Laboratories, Melbourne.

FIGURE 1. — EXPERIMENTAL DESIGN


C3H
sera titrated
sera titrated
C57BL
sera titrated
sera titrated
C3H and C57BL mice injected i/p with diphtheria toxoid.
Spleens removed 40hrs 6 days, and 7 days after secondary antigenic stimulation.
Whole cells and lysed-cell homogenates transferred both isologously and homologously.
Antigenic challenge 11–12 weeks after transfers.

pooled within the strains. The tissues were then finely diced with sharp surgical scissors for five to ten minutes to release the cells. The cellular preparations thus obtained were washed twice in Earle's saline by centrifugation at 700 r.p.m. in an angle centrifuge for five minutes, and the sedimented cells resuspended by gentle pipetting with a wide-mouthed Pasteur pipette. After the second washing the cells were suspended in the requisite volume of Earle's saline so as to yield a whole-cell concentration of 35×10^6 cells per ml.

Lysed-cell homogenates. The lysed-cell homogenates were simply prepared by freezing and thawing the whole-cell suspensions three times in a dry ice-alcohol mixture and re-suspending the sub-cellular components and debris after each thawing with the aid of a mechanically-driven Potter-Elvehjem homogeniser.

The collection and titration of sera.

The mice were bled from the tail vessels and usually 0·2 to 0·4 ml. of blood was obtained at each bleeding. After allowing the blood to clot at room temperature, 0·1 ml. of serum was drawn off after standing the specimens at 6° to 8° C. overnight in the refrigerator. The sera were then diluted 1/10 in saline and stored in a deep-freeze at — 20° C. until required for titration. All specimens were titrated retrospectively.

Antibodies were titrated by Fisher's modification of the Boyden tanned-cell technique (Fisher, 1952).

Control of the sensitivity of titration.

As the degree of sensitisation of the tanned cells varied between batches an hyper-immune rabbit serum was titrated in the presence of each batch of sensitised erythrocytes. The titre of the hyper-immune serum usually varied between 30×10^3 and 60×10^3 and the titres of the mouse sera were corrected accordingly (30×10^3 being taken as the base level).

The possible influence of the sensitivity of titration as a potential source of error upon interpretation of the results was avoided by titrating sera from treated groups and the appropriate control mouse sera with the same batch of sensitised cells. Subsequent analyses are based upon direct comparison of these serum antibody levels.

RESULTS.

Preliminary experiment.

Little or no antibody was detected in the sera of the mice of both strains within three weeks of primary antigenic stimulation, while antibody was detected in the sera of 3/6 mice of the C3H strain and 6/6 mice of the C57Bl strain at three days following secondary stimulation. All the mice of both strains had responded with serum antibody titres by the sixth day after secondary antigenic stimulation. The C57Bl mice responded more vigorously, earlier and with higher serum titres, than the mice of the C3H strain.

Experiment 1.

Cells from spleens harvested from both C3H and C57Bl strains of mice were transferred isologously and homologously six days after secondary antigenic challenge of the donor animals. The antibody levels given in Table 1 were titrated in the sera of the recipient mice at intervals of time following antigenic challenge 11 weeks after transfer. Serum titres of antibody of recipient groups following lysed-cell homogenate transfer are not shown either in Table 1 or in subsequent tables, as the responses observed in these groups did not differ significantly from those observed in the appropriate control groups.

The results given in Table 1 show that antigenic stimulation produced clearly defined secondary responses in both strains following isologous whole-cell transfer (groups 1 and 3), while the serological response obtained in the groups following homologous whole-cell transfer did not differ from the responses observed in the appropriate control groups (group 2 cf. group 6, group 4 cf. group 5).

Experiment 2.

Spleen cells were transferred both isologously and homologously 40 hours after secondary antigenic challenge of the donor animals. The serum antibody levels of the recipient animals were recorded at two-day intervals following transfer and after antigenic challenge given 12 weeks following transfer. The antibody levels detected in the sera of the recipient groups immediately following transfer are given in Table 2 and the titres observed in the sera of these animals following antigenic challenge are shown in Table 3.

The results of the isologous transfer of C3H spleen cells (Group 1) and homologous transfer of both strains of cells (Groups 2 and 4) have not been included in Table 2, as antibody was not detected in the sera of these groups

TABLE 1.

**Transformed titres of serum antibody in recipients after antigenic challenge given 11 weeks after transfer.*

Treatments	Mouse	Days after antigenic stimulation														
		0	1/2	3/4	5/6	7/8	9/10	11/12	14/15	16/17	18/19	21/22	23/24	28	35	63
Group 1 C3H isologous	1	⩽1	†⩽1	⩽1	2·0	1·5	7·5	8·5	5·0	7·5	8·5	9·5	9·5	6·5	8·0	5·0
	2	⩽1	⩽1	⩽1	2·0	7·5	9·5	10·5	10·0	9·0	10·5	11·0	>11·0	8·0	8·0	7·0
	3	⩽1	⩽1	⩽1	1·0	7·5	9·5	10·5	10·0	8·5	9·5	11·0	8·5	8·5	9·0	8·0
	4	⩽1	⩽1	⩽1	5·0	8·5	9·0	11·5	>10·5	>10·5	>11·0	>11·0	>11·0	9·5	9·0	—
	5	⩽1	⩽1	⩽1	6·0	9·5	9·5	11·0	10·5	10·0	10·5	11·0	11·0	8·5	9·5	9·0
	6	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	⩽1	1·0	3·0
	‡ Av.	⩽1	⩽1	⩽1	3·0	7·0	9·0	10·5	9·0	9·0	10·0	10·5	10·0	8·0	8·5	7·5
Group 2 C3H homologous (C57Bl recipients)	1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	2·5	non-	specific	death	—	—	—
	2	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	2·5	3·5	5·5	5·0	4·0	3·5	4·0
	3	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·5	2·5	3·5	3·5	2·5	3·0	4·0
	4	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	2·0	3·0	5·0	4·0	2·0	4·5	6·0
	5	⩽1	⩽1	⩽1	⩽1	⩽1	1·5	1·5	1·0	2·0	4·0	3·0	2·5	2·5	5·0	5·5
	6	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	1·5	3·0	3·5	4·0	3·0	3·0	5·0
	Av.	⩽1	⩽1	⩽1	⩽1	< 1	⩽1	⩽1	0·5	2·0	3·0	4·0	4·0	3·0	4·0	5·0
Group 3 C57Bl isologous	1	⩽1	⩽1	⩽1	⩽1	7·5	9·5	11·0	11·5	10·0	11·0	10·0	>11·0	9·0	9·0	8·0
	2	⩽1	⩽1	⩽1	1·5	9·5	10·5	11·5	>10·5	10·5	>11·0	11·0	>11·0	10·0	10·5	9·0
	3	⩽1	⩽1	⩽1	1·0	8·5	10·0	12·0	>10·5	5·5	4·5	11·0	>11·0	9·5	9·0	8·0
	4	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	3·0	4·5	4·0	3·5	5·0	5·5
	5	⩽1	⩽1	⩽1	6·5	8·5	10·0	10·5	10·0	9·5	10·5	11·0	11·0	9·5	8·5	8·0
	6	⩽1	⩽1	⩽1	6·5	9·0	11·5	11·0	9·5	9·5	11·0	11·0	10·5	9·0	8·5	7·0
	Av.	⩽1	⩽1	⩽1	3·0	8·5	10·5	11·0	10·5	9·0	9·5	11·0	11·0	9·5	9·0	8·0
Group 4 C57Bl homologous (C3H recipients)	1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0
	2	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	1·0	1·0	2·0	2·5
	3	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	⩽1	1·0	3·0
	4	⩽1	⩽1	⩽1	⩽1	⩽2	⩽1	⩽1	⩽1	⩽1	2·0	2·0	1·5	1·0	2·0	5·5
	5	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	1·0	1·0	1·0	⩽1	4·0
	6	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0
	Av.	⩽1	⩽1	⩽1	⩽1	⩽2	⩽1	⩽1	⩽1	⩽1	0·5	1·0	1·0	0·5	1·0	3·0
Controls (untreated) Group 5 C3H strain	1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	2·5	2·5	4·0	6·5	7·5
	2	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	4·5	⩽1	⩽1	⩽1	⩽1
	3	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·5	⩽1	1·0	6·0	8·0
	4	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	⩽1	⩽1	⩽1	3·5
	5	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	0·5	1·0	⩽1	⩽1	1·0	3·5
	6	⩽1	⩽1	⩽1	⩽1	⩽2	⩽1	⩽1	0·5	⩽1	3·0	4·0	4·0	4·0	6·5	5·5
	Av.	⩽1	⩽1	⩽1	⩽1	⩽2	⩽1	⩽1	⩽1	⩽1	1·0	2·5	1·0	1·5	3·5	4·5
Group 6 C57Bl strain	1	⩽2	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	0·5	1·5	4·5	3·0	4·0	1·5	5·0	6·0
	2	⩽2	⩽1	⩽2	⩽2	⩽1	⩽1	⩽1	⩽1	⩽1	1·5	2·0	4·5	2·5	5·0	4·5
	3	⩽2	⩽1	⩽2	⩽1	⩽1	⩽1	⩽1	⩽1	0·5	1·5	2·0	3·5	3·0	5·5	6·0
	4	⩽2	⩽1	⩽1	⩽2	⩽1	⩽1	⩽1	0·5	1·0	3·0	3·0	4·5	4·5	4·5	7·0
	5	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	2·5	3·5	—	3·0	4·0	3·5
	Av.	⩽2	⩽1	⩽2	⩽2	⩽1	⩽1	⩽1	⩽1	0·5	2·5	2·5	4·0	3·0	5·0	5·5

* The data have been reduced by transforming the reciprocals of the end-point dilutions of the titrations according to the expression $y = 1 + \log_2 x/10$, where y = transformed value and x = reciprocal of the end-point dilution.

† Generally serial two-fold dilutions were prepared from an initial 1/10 dilution of serum.

‡ Averages are estimated from pools of the transformed titres of the 6 animals in each group over a 2 day interval, excepting for the periods 28, 35 and 63 days.

TABLE 2.

Transformed titres of serum antibody recipients following cell transfer.

Group 3	Mouse	Days after transfer																
		−1	0	1	2	3	5	7	9	12	14	16	19	22	26	47	84
C57Bl isologous whole-cell transfer	1	<1	Day of Transfer	<1	<1	<1	2·0	2·5	2·0	2·5	2·5	4·0	5·0	4·0	3·5	2·5	<1
	2	<1		<1	<1	1·0	2·5	1·5	3·0	1·5	2·0	2·5	3·5	3·5	2·0	1	<1
	3	<1		<1	<1	1·0	2·5	1·0	1·5	1·5	1·5	2·0	2·5	3·5	1·5	2·0	<1
	4	<1		<1	<1	0·5	2·0	1·0	1·0	1·5	1·2	2·2	1·0	2·0	1·5	2·0	<1
	5	<1		<1	<1	0·5	2·0	1·5	1·5	1·0	2·0	3·5	3·5	2·5	2·5	2·0	<1
	6	<1		<1	<1	1	1·5	1·0	1·0	1·0	2·0	3·0	3·5	2·5	1·5	1·0	<1
	Av.	<1		<1	<1	0·5	2·0	1·5	1·5	1·5	1·5	2·5	3·0	3·0	2·0	1·5	<1

TABLE 3.

Transformed titres of serum antibody in recipients following antigenic challenge given 12 weeks after transfer.

Treatments	Mouse	Days after antigenic stimulation													
		0	1/2	3/4	5/6	7/8	9/10	11/12	14/15	16/17	18/19	21/22	28	42	56
Group 1 C3H isologous	1	⩽1	⩽1	⩽1	⩽1	6·0	10·5	11·0	11·0	11·0	11·0	11·0	9·0	10·5	10·5
	2	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	1·0
	3	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	5·0	5·0	6·0	4·5	4·5	5·5	4·5
	4	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	2·0	3·0	3·5
	5	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·5	⩽1	⩽1	⩽1	⩽1	1·0	1·0
	6	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	3·0	⩽1	2·5	3·5	1·5	2·0	3·0
	*Av.														
Group 2 C3H homologous (C57Bl recipients)	1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·5	2·0	4·0	3·5
	2	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	2·0	4·0	4·5
	3	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	2·0	4·0	4·0	5·0	7·5	7·5
	4	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	2·0	2·5	3·0	3·5	3·5	5·0	5·5
	5	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·5	2·0	2·0	2·0	3·5	3·5	6·5	6·0
	6	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·5	6·0	6·5
	Av.	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	0·5	⩽1	⩽1	1·5	2·0	3·0	5·5	5·5
Group 3 C57Bl isologous	1	⩽1	⩽1	⩽1	⩽1	8·0	11·0	11·5	7·5	11·0	11·0	8·5	8·5	10·5	8·5
	2	⩽1	⩽1	⩽1	⩽1	9·0	10·5	11·5	8·0	11·0	11·0	10·0	8·5	8·5	7·0
	3	⩽1	⩽1	⩽1	⩽1	7·5	9·0	11·0	8·0	10·0	10·5	9·0	9·0	9·5	8·5
	4	⩽1	⩽1	⩽1	4·0	12·0	12·0	12·0	11·0	11·0	11·0	11·0	10·0	10·0	9·0
	5	⩽1	⩽1	⩽1	2·0	12·0	12·0	12·0	11·0	11·0	10·5	11·0	11·0	10·5	9·0
	6	⩽1	⩽1	⩽1	3·0	8·5	8·5	9·0	9·0	8·5	8·0	6·5	6·5	6·5	4·5
	Av.	⩽1	⩽1	⩽1	1·5	9·5	10·5	11·0	9·0	10·5	10·5	9·5	9·0	9·5	8·0
Group 4 C57Bl homologous (C3H recipients)	1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	4·0	6·5
	2	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	3·0	3·0
	3	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	5·0	7·5
	4	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	2·0	4·0	5·0
	5	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	4·5	5·5
	6	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	2·0	4·5
	Av.	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	0·5	4·0	5·5
Controls—Group 5 C3H strain	1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1
	2	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	1·5	3·5	4·5	6·0
	3	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	3·0	4·5
	4	⩽2	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	0·5	1·0	2·0	3·5	4·5
	5	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	0·5	0·5	4·0	5·0
	6	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	0·5	4·5	5·0
	Av.	⩽2	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	0·5	0·5	1·5	3·0	4·0
Group 6 C57Bl strain	1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	0·5	1·0	3·0	3·0	3·5	3·5
	2	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	0·5	1·5	2·0	3·0	2·5	5·0	7·0
	3	⩽1	⩽2	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	2·0	2·0	3·5	5·5	7·0
	4	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	1·0	2·0	3·5	5·5	5·5	5·0
	5	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	2·0	2·0	5·0	3·0	3·0	4·5	9·0	10·5
	6	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	⩽1	1·0	0·5	1·5	3·0	4·5	5·5
	Av.	⩽1	⩽2	⩽1	⩽1	⩽1	⩽1	0·5	0·5	1·5	2·0	2·5	3·5	5·5	6·5

* Calculation of averages for Group 1 is not meaningful as Mouse 1 and possibly Mouse 3 are exhibiting secondary responses while the remaining animals in the group are giving primary immunological responses.

immediately following transfer. The results given in Table 2 show, however, that serum antibody was detectable in 4/6 mice as early as three days following isologous transfer of stimulated C57Bl spleen cells (Group 3) and in all animals of the group during the period five to 26 days following transfer. It is interesting to note that antibodies were still detectable in the sera of the recipients 47 days after transfer.

The results given in Table 3 show that a clearly defined secondary response was produced in all six animals of the C57Bl, isologous-transfer group following antigenic challenge, while only one animal of the C3H isologous-transfer group manifested this response.

Experiment 3.

Spleen cells were transferred both isologously and homologously among the C3H and C57Bl strains seven days after secondary challenge of the donor animals. The serum antibody levels of the recipient animals were titrated during the period immediately following transfer, and following antigenic challenge given 12 weeks after transfer.

The serum levels of antibody in the recipients immediately following isologous whole-cell transfer are shown in Table 4. As antibodies were not detected in the sera of the animals following homologous whole-cell transfer these negative results have not been recorded.

The results of this study clearly demonstrate that antibodies are detectable in the sera of the recipients when isologous whole-cells, alone, are transferred. As antibodies are already detectable in the sera of the C57Bl recipients at two days after transfer, while antibodies were not detected in the sera of the C3H recipients of homologous C57Bl whole-cell transfer, it is concluded that homologous discrimination against the C57Bl cells has occurred within two days following transfer.

The levels of serum antibody titrated in the recipients of isologous and homologous whole-cell transfer following antigenic challenge given 12 weeks after transfer are shown in Table 5.

The results given in Table 5 show that the recipients of isologously transferred cells have generally responded more vigorously than the appropriate control groups (group 1 cf. group 5, group 3 cf. group 6), while the recipients of homologously transferred cells have not responded more vigorously than the controls. It is interesting to note that in this experiment, although not in experiment 1, the response elicited by antigenic challenge in the recipients of C57Bl spleen cells was not so vigorous as the response observed in the C3H isologous transfer groups. This result is surprising, as previous experience has established that C57Bl mice are more immunologically reactive to diphtheria toxoid than the C3H strain, and the results of the present experiment have clearly shown that the C57Bl donor cells produced antibodies more rapidly and more consistently than the C3H donor cells immediately following transfer (see Table 4).

TABLE 4.

Transformed titres of serum antibody in recipients following cell transfer.

		Days after transfer															
	Mouse	−1	0	1	2	3	4	6	8	10	13	15	17	20	24	27	73
Group 1 C3H isologous whole-cell transfer	1	<2	Day of transfer	<2	<1	<1	<1	<1	1·5	2·0	1·5	1·0	1·0	0·5	<1	<1	<2
	2	<2		<1	<1	<1	<1	<1	<1	1·5	1·0	<1	<1	<1	<1	<1	<1
	3	<2		<2	<1	<1	<1	<1	2·0	3·0	2·5	2·0	1·0	<1	1·0	2·0	<1
	4	<2		<1	<1	<1	<1	<1	0·5	1·5	1·0	<1	<1	<1	<1	<1	<1
	5	<2		<2	<1	<1	<1	<1	0·5	1·5	<1	<1	<1	<1	<1	<1	<1
	6	<1		<1	<1	<2	<1	1·5	2·5	2·5	3·0	3·0	2·5	2·5	2·0	2·0	<1
	Av.	<2		<2	<1	<2	<1	0·5	1·0	2·0	1·5	1·0	1·0	0·5	0·5	0·5	<2
Group 3 C57Bl isologous whole-cell transfer	1	<1	Day of transfer	<2	2·0	1·5	1·0	3·0	2·5	3·0	1·0	1·0	<2	<1	<1	<1	<1
	2	<2		<1	1·0	1·0	<1	<1	0·5	1·0	<1	<1	<1	<1	<1	<1	<1
	3	<1		<1	1·0	1·5	0·5	2·5	<1	3·5	3·0	2·0	<2	<1	<1	<1	<1
	4	<1		<1	1·0	1·0	1·5	2·5	2·5	3·0	2·5	1·5	2·0	0·5	<1	<1	<1
	5	<1		<1	1·0	1·0	1·0	2·0	2·0	3·0	3·0	2·0	<3	0·5	<1	<1	<1
	6	<1		<1	1·5	1·0	1·0	2·5	3·0	3·0	2·5	2·5	1·5	1·0	<1	<1	<1
	Av.	<2		<2	1·5	1·0	1·0	2·0	2·0	3·0	2·0	1·5	0·5	0·5	<1	<1	<1

TABLE 5.

Transformed titres of serum antibody in recipients following antigenic challenge given 12 weeks after transfer.

Treatments	Mouse	Days after antigenic stimulation													
		0	1/2	3/4	5/6	7/8	9/10	11/12	14/15	16/17	18/19	21/22	29	43	57
Group 1 C3H isologous	1	<1	<1	<1	<1	5·5	>10·0	9·0	9·5	10·0	>12·0	>12·0	8·5	7·5	9·5
	2	<1	<1	<1	<1	3·0	>10·0	6·0	6·5	6·5	8·5	8·5	<1	<1	7·5
	3	<1	<1	<1	<1	5·5	>10·0	8·0	10·0	9·0	12·0	>12·0	7·0	–	8·0
	4	<1	<1	<1	5·0	7·0	9·0	8·5	9·0	9·0	12·0	12·0	9·5	4·5	9·5
	5	<1	<1	<2·5	<1	4·0	9·0	8·0	9·0	10·0	>12·0	>12·0	9·5	9·0	>10·0
	6	<1	<1	<1	5·0	8·0	>10·0	10·0	10·5	11·5	>12·0	–	10·5	8·5	>10·0
	Av.	<1	<1	<2·5	1·5	5·5	9·5	8·5	9·0	9·5	11·5	9·5	6·5	6·0	9·0
Group 2 C3H homologous (C57Bl recipients)	1	<1	<1	<1	<1	<1	<1	<1	2·0	2·0	4·0	5·0	5·0	9·0	8·0
	2	<1	<1	<1	<1	<1	<1	<2	3·0	4·0	5·5	5·0	6·0	5·0	6·0
	3	<1	<1	<1	<1	<1	<1	<1	2·0	3·5	4·5	4·0	3·5	5·0	6·0
	4	<1	<1	<1	<1	<1	–	1·0	1·0	2·5	2·5	3·5	2·5	4·5	6·0
	5	<1	<1	<1	<1	<1	–	<1	2·0	3·0	5·5	5·0	2·5	5·5	7·5
	6	<1	<1	<1	<1	<1	–	1·0	<1	1·5	3·0	3·0	1·0	3·0	5·5
	Av.	<1	<1	<1	<1	<1	<1	0·5	1·5	3·0	4·0	4·5	3·5	5·5	6·5
Group 3 C57Bl isologous	1	<1	<1	<1	<1	<1	<1	<1	1·0	3·0	5·0	4·5	3·0	4·0	5·0
	2	<1	<1	<1	<1	<1	<1	<1	1·5	1·5	3·5	3·5	<1	0·5	2·0
	3	<1	<1	<1	<1	<1	3·5	5·0	5·5	5·5	8·0	6·5	1·5	1·5	4·5
	4	<1	<1	<1	<1	<1	3·0	2·5	2·0	3·0	5·0	5·0	2·0	6·0	6·0
	5	<1	<1	<1	<1	<1	<1	6·0	2·0	3·0	5·0	4·5	2·5	6·0	6·0
	6	<1	<1	<1	<1	2·5	6·5	6·5	6·5	6·0	8·0	8·0	3·0	3·5	7·5
	Av.	<1	<1	<1	<1	0·5	2·0	3·5	3·0	3·5	6·0	5·5	2·0	5·5	5·0
Group 4 C57Bl homologous (C3H recipients)	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
	2	<1	<1	<1	<1	<1	<1	<1	<1	<1	1·5	2·0	<1	2·0	4·5
	3	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	1·0
	4	<1	<1	<1	<1	<1	<1	<1	<1	<1	1·0	1·0	<1	<1	2·5
	5	<2	<1	<1	<1	<1	<1	<1	<1	1·0	1·5	3·0	0·5	<1	4·0
	6	<1	<1	<1	<1	<1	<1	<1	<1	1·0	2·5	3·5	<1	<1	2·5
	Av.	<2	<1	<1	<1	<1	<1	< 1	<1	0·5	1·0	1·5	<1	0·5	2·5
Controls—Group 5 C3H strain	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	1·0	2·0	0·5	2·5	6·5
	2	<1	<1	<1	<1	<1	<1	<1	<1	<1	2·5	3·0	<1	1·5	4·5
	3	<1	<1	<1	<1	<1	<1	<1	<1	<1	1·0	2·0	0·5	4·0	>10·0
	4	<1	<1	<1	<1	<1	<1	<1	<1	<1	2·5	3·0	<1	5·0	>9·5
	5	<1	<2	<1	<1	<1	<1	<1	<1	<1	2·0	3·5	2·5	5·5	>8·5
	6	<1	<1	<1	<1	<1	<1	<1	<1	1·5	3·5	3·5	<1	2·5	5·5
	Av.	<1	<2	<1	<1	<1	<1	<1	<1	0·5	2·0	3·0	0·5	3·5	7·5
Group 6 C57Bl strain	1	<1	<1	<1	–	<1	<1	<1	1·0	3·0	4·5	5·5	2·0	4·0	6·5
	2	<1	<1	<1	–	<1	<1	<1	2·5	2·0	3·5	5·0	3·0	6·0	7·5
	3	<1	<1	<1	<1	<1	<1	<1	3·0	4·5	6·5	7·5	3·5	5·5	7·0
	4	<1	<1	<1	<1	<1	–	<1	1·0	1·0	2·5	3·0	2·5	4·0	6·5
	5	<1	<1	<1	<1	<1	<1	<1	<1	1·5	3·0	3·5	2·0	6·0	9·0
	6	<1	<1	<1	<1	<1	<1	<1	1·5	1·0	4·0	5·0	3·0	4·0	5·5
	Av.	<1	<1	<1	<1	<1	<1	<1	1·5	2·0	4·0	5·0	2·5	5·0	7·0

DISCUSSION.

The most impressive feature of the present studies is that accelerated response following subsequent antigenic challenge, was confined to the recipients of isologous whole-cell transfer. The results of our experiments therefore underline the advantage to be gained by the use of homozygous animals for the purpose of cell-transfer studies. The failure of the recipient groups of mice to show a secondary response following antigenic challenge some 11 or 12 weeks after homologous cell transfer is undoubtedly due to the rejection of the transplanted cells by the appropriate immunological response. This result is anticipated by the early studies of Topley (1930), who found that antigenic stimulation of rabbits following transfer of spleen cells from immunised donors produced a primary response. Recently, Stavitsky, Axelrod and Pruzansky (1957) have studied antibody production by homotransplanted lymphoid tissues and cells in the rat. They, too, failed to obtain a secondary response in the recipient animals by antigenic challenge following transfer.

Neither homologous whole cells nor isologous and homologous homogenates influenced the response of the recipients to the challenge inoculation. The results obtained during the present studies cannot therefore be attributed to the transfer of native antigen, and must depend on the implantation and survival of immunologically competent cells. It is noteworthy that the absence of any effect in the recipient of residual antigen from the donor was apparent when the spleen cells were transferred as early as 40 hours after secondary stimulation of the donor animals. This undoubtedly reflects the effectiveness of accelerated immune elimination of native antigen in the donors (Sorkin and Boyden, 1959).

Antibodies were detected in the sera of recipient animals shortly after isologous transfer of C57Bl spleen cells, while antibodies were not detected in the sera of the recipients following homologous transfer of the same cell preparation. Titres were low, but there was a consistent difference from the homologous recipients. On the other hand, the less reactive C3H mice showed little or no evidence of difference between isologous and homologous recipients and no appearance of antibody in either group before six or seven days. We cannot, on present evidence, eliminate the possibility that the failure of antibody to appear in C3H recipients after transfer of C57Bl cells was due to some non-immunological factor in C3H mice.

Nevertheless, in all discussion of this type of experiment by others it has been the rule to ascribe differences to histocompatibility (immunological) factors. We may, therefore, adopt the provisional hypothesis that a homograft-like rejection mechanism is operative following homologous transfer either at the cellular level, causing complete cell destruction, or at the sub-cellular level, inhibiting cellular function. The results of experiment 2 indicate that rejection is effective within three days of transfer of the homologous cells, while this upper time

limit is reduced to two days in experiment 3. This phenomenon is in sharp contrast with the results of the studies described by Harris, Harris and Farber (1954), who have demonstrated the production of circulating antibodies by homotransplanted lymphoid cells in the heterozygous rabbit – a situation which might be supposed to be analogous to that following the homotransplantation of spleen cells between strains of mice in our studies. It is reasonable to assume that host discrimination against the foreign cells is either mediated by a homograft reaction of the type elucidated by the studies of Medawar and his colleagues, or by specific elimination by iso-antibodies of the type described by Gorer and O'Gorman (1956), and more recently by Boyse (1959). First-set homograft rejection has been observed as early as 24 to 48 hours after transplantation of scales in the goldfish (Hildemann, 1956) and host rejection of homologously transferred spleen cells in the mouse was detected as early as three days following transfer in Boyse's experiments. Medawar and his colleagues, using an elegant technique involving the cellular transfer of delayed hypersensitivity to homotransplantation antigens, have observed that specific sensitisation of the peripheral blood leucocytes in the guinea pig is acquired within 48 hours of antigenic injection (Medawar, personal communication). The feature of these reports, and of our studies, is that the upper time limit for the acquisition of immunological reactivity, alone, is defined. Thus the latent period given by these studies for the acquisition of responsiveness may be a function of the sensitivity of technique rather than an inherent characteristic of the immune response. Hildemann (1956) has pertinently drawn attention to Jerne's studies (Jerne, 1956), which demonstrated specific antibody production within 48 hours of injection of the horse with T4 phage antigens. This current trend of reducing the latent period necessary for the acquisition of immunological responsiveness along a contracting time-scale which may eventually extrapolate to zero, lends support both to Jerne's original "natural selection" hypothesis (Jerne, 1955) and to Burnet's concept of "clonal selection" (Burnet, 1957).

Acknowledgments. We are much indebted to Sir Macfarlane Burnet, O.M., F.R.S., for his advice and guidance during the course of these investigations. In addition, we gratefully acknowledge the excellent technical assistance given to us by Miss Rae Brady and Miss Margaret McElroy.

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LONG TERM STORAGE OF TISSUE CULTURE CELLS

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(*Accepted for publication 5th July, 1960.*)

SUMMARY.

Survival rates after storage at — 65° C. for periods ranging from 1-9 months are given for 15 established cell lines of epithelial appearance from a wide variety of sources. Recently isolated epithelial cells from monkey kidney and human amnion tissue and fibroblast-like cells recently isolated from cervical biopsies have also shown suitable survival rates on storage at — 65° C. All cultured cells are still viable after nine months, although a variation in recovery rate is shown from cell type to cell type. Freshly isolated monkey kidney and human amnion cells which have not been cultured fail to survive in significant numbers when stored at — 65° C. The number of cells surviving is dependent on the concentration of cells stored and on the rate of thawing.

INTRODUCTION.

A satisfactory method for storing tissue culture cells over long periods is necessary for several reasons. The continuous subculture of a comprehensive range of cell lines involves a large amount of work as well as the risk of loss through bacterial contamination. There is also the possibility of an alteration of the cell type under investigation due to selection or chance mutation. Furthermore, a source of primary cell material may not be available when required for a particular study.

Investigations have been made by Scherer and Hoogasian (1954) on the storage of HeLa and L strain cells at — 70° C. and by Swim *et al.* (1958) on the survival after freezing of a number of fibroblast cells, both long established and recently isolated lines. In this paper the sensitivity to prolonged storage of a large range of established epithelial cell lines, as well as freshly isolated epithelial and fibroblast-like cells, will be reported.

Materials and Methods.

A total of 15 established cell lines of epithelial-like appearance obtained from a wide variety of sources (Table 1) were maintained in this laboratory for several months on medium 199 (Morgan, Morton and Parker, 1950) containing 20 p.c. serum, usually calf serum, before being used in storage experiments. Trypsinised monkey kidney cells were grown in medium 199 containing 5 p.c. calf serum. Amnion cells were obtained by the method of Ferguson and Tobin (1958) and cultured in medium 199 containing 20 p.c. pooled human serum. Primary fibroblast cultures were prepared by the addition of 0·25 p.c. trypsin to small biopsy samples then adding to the suspension of cells a growth medium comprising 199 + 20

p.c. foetal calf serum. The cells were grown in 60 mm. petri dishes in an incubator continuously flushed with a 5 p.c. CO_2 in air mixture. For storage the cells were removed by 0·25 p.c. trypsin washed once in phosphate buffered saline and counted in a haemocytometer. The cells were then suspended in their normal growth medium plus 10 p.c. Merck reagent grade glycerol to give a concentration of approximately 2×10^6 cells per ml. 1 ml. aliquots of this suspension were distributed in ampoules, sealed and placed in a mechanical — 65° C. freezing cabinet. For sub-culture, the ampoules were thawed in a 37° C. water bath immediately on removal from the freezing cabinet. After 15 minutes the cells were taken from the ampoule which was then rinsed to ensure that all cells had been removed. The cell suspension was washed and spun three times in growth medium pre-heated to 37° C. All traces of glycerol were thus removed before placing the cells in a milk dilution bottle with the appropriate growth medium. After incubation for 17 hours at 37° C. the cells which had attached to the glass surface were removed with trypsin and counted. The cells were then transferred to fresh bottles containing growth medium for further incubation in order to observe their cultural appearance.

RESULTS.

Survival rates of continuous cell lines after various periods of storage are shown in Table 1. Although the figures vary somewhat from cell line to cell line, it can be seen that in the majority of cases a large number of cells is still viable after 6-9 months. Two exceptions are J.111 and F.R.M. which show a low survival rate. However, these cells, along with all the others listed in Table 1, gave a normal healthy appearance on subculture.

Cells recently established in tissue culture were also stored at − 65° C. (Table 2). The amnion and monkey kidney cells were stored by two methods: (1) cells obtained by trypsination of the particular tissue were washed three times in phosphate buffered saline (Dulbecco and Vogt, 1954), then stored immediately; (2) cells grown for one passage on glass before being stored away. The cells which survived the first method grew as healthy epithelium on subculture, but were not numerous enough to be reliably counted. Better results were obtained with the second method, though the recovery rate was not as high as with the continuous cell lines of Table 1.

The fibroblast cells which had been passed from 6-8 times before storage gave the survival rates shown in Table 2 and had a normal appearance when examined on subculture.

The effect of the rate of freezing and thawing was determined by making a comparison of two methods of freezing: (1) ampoules were placed directly into the − 65° C. cabinet and allowed to freeze; (2) ampoules were frozen more rapidly by immersing them in an alcohol-dry ice bath at − 78° C. before being stored away in the freezing cabinet. The second method was discontinued as approximately only half the number of viable cells could be recovered as compared with the first method.

Three methods for thawing the ampoules were tested and the results are shown in Table 3. The most satisfactory procedure was to place the ampoules

TABLE 1.

Survival of continuous cell lines after prolonged storage at −65° C.

Cell line	Tissue of origin	Reference	p.c. Cells surviving after storage time (in months)				
			1	2	3	6	9
HeLa	Carcinoma of cervix	Scherer, Syverton and Gey (1953)	85		73		42·3
K.B.	Pharyngeal epithelioma	Eagle (1955)			46·1	29·2	
J.111	Leukemia	Osgood and Brook (1955)			53	0·9	
H.Ep 2	Human nasopharynx	Toolan (1954)	41		17		
Detroit 6	Bone marrow of patient with carcinoma of lung	Berman and Stulberg (1956)			67	10·5	
Detroit 34	Bone marrow of patient with carcinoma of bone	Berman and Stulberg (1956)			30	15·9	
Gut	Human gut	(Unpublished)		66			
Appendix	Human appendix	Chang (1954)	69		44	13·4	
Conjunctiva	Human conjunctiva	Chang (1954)			61	27	
Monkey Heart	Cynomolgous monkey heart	Salk and Ward (1957)		80	78		14·2
Swine Kidney	Swine kidney	Madin (Unpublished)	59		24		
F.R.M.	Rabbit kidney	Tytell (Unpublished)	38		32		0·9
C.3	Benign myofibroma	Salk (Unpublished)			50	29·3	
Human Kidney PIR	Human kidney	White (Unpublished)			30		22·1
Human Kidney MIL	Human kidney	White (Unpublished)			27		

in 37° C. water bath for 15 minutes. This gave an appreciably greater recovery rate than keeping the ampoule for three minutes in 37° C. water bath or by leaving the ampoule at room temperature until thawed.

The age of cells was not found to be critical, as no appreciable differences have been noted following storage of cells grown for varying lengths of time provided the cultures used for storage had a healthy appearance.

TABLE 2.

Survival of primary cell cultures after prolonged storage at −65° C.

Tissue	Cell type	Passage No.	p.c. Cells surviving after storage time (in months)			
			1	2	3	6
Human amnion	Epithelial	0	V		V	
Human amnion	Epithelial	1	26		16	15·4
Monkey kidney	Epithelial	0	V		V	
Monkey kidney	Epithelial	1	13		10	0·5
Cervical biopsy	Fibroblast	8	15	8·2		
Cervical biopsy	Fibroblast	6	47·2	7·6		
Cervical biopsy	Fibroblast	8	30·6	9·8		

V = viable cells present but not in sufficient number for reliable counting.

TABLE 3.

Effect of rate of thawing on percentage recovery of viable cells.

Cell line	p.c. Cells surviving after 3 months' storage		
	Cells thawed in 37° C. water bath, 15 mins.	Cells thawed in 37° C. water bath, 3 mins.	Cells thawed room temperature
HeLa	41·7	36·1	28·7
Monkey heart	64·5	53·5	32·0
K.B.	30·5	19·6	11·9

TABLE 4.

Effect of dose on survival of cells.

Cell line	No. of cells per ampoule $\times 10^6$	p.c. Cells surviving after storage time (in months)	
		3	9
HeLa	9·0	24	1·8
	2·2	73	42·3
Miles	10·3	0·7	0·02
	2·0	27	
Pirani	9·7	4·9	
	2·1	30	22·1

The effect of dose on survival was also investigated. Cells were suspended in 1 ml. of growth medium and placed in an ampoule at concentrations of approximately 2×10^6 per ml. and 10×10^6 per ml. As can be seen from Table 4, a significantly better result is obtained at the level of 2×10^6 cells per ampoule.

DISCUSSION.

The results show that it is possible to store for long periods a wide range of established cell lines at present being used in virus and tissue culture studies. These cells do not appear to have altered with regard to their morphology, nutritional requirements or virus susceptibility. Recently isolated epithelial-like cells grown in tissue culture show a somewhat lower survival rate. However, the cells do survive in sufficient quantity to make this a very convenient method for storing primary tissue culture material for short periods until it is required for a particular study. It is essential, however, that these cells should be passaged at least once on glass before being stored. Fibroblast-like cells, after a few passages, appear to survive storage to about the same degree as the epithelial-like cells. A strain of fibroblast cells which had undergone 18 passages before storage showed a 39·9 p.c. survival rate after three months and showed no alteration in cultural appearance, growth rate, or chromosome number.

These studies describe a simple method for the efficient storage of cells which would be suitable for most tissue culture and virus laboratories. There are indications (Hauschka, 1959) that higher survival rates could be expected if special equipment is used. This involves the use of apparatus which regulates the lowering of temperature of the ampoules by one degree per minute down to $-25°$ C. before being placed in a dry-ice-alcohol cabinet permanently held at $-78°$ C. We have found that the concentration of cells in an ampoule affects the result (Table 4), this presumably being due to the difference in the rate of freezing of these ampoules.

Glycerol plays an important function in the freezing process. It prevents dehydration due to the formation of ice crystals because of its ability to bind water strongly. Swim, Haff and Parker (1958) have noted that the concentration of glycerol needed for optimal preservation varies from cell line to cell line. Although we have used a constant concentration of 10 p.c. as a matter of routine convenience, higher survival figures presumably could have been obtained if glycerol requirements for each particular cell had been investigated fully. As it had been found that a highly purified glycerol must be used, Merck reagent grade was used throughout all these experiments.

Thawing has been found to be an important stage. When cells are frozen at the temperature and concentration of glycerol described, the formation of ice crystals of any appreciable size is prevented. On thawing, ice crystals can grow rapidly at temperatures near the melting point making it necessary to pass through this danger region as rapidly as possible. This is achieved by placing

the ampoules in a 37° C. water bath and taking particular care that the temperature of the water bath does not fall due to the addition of the frozen material. Allowing the ampoules to remain at 37° C. for a short period after thawing is complete appears to be beneficial. The slower process of thawing the ampoules at room temperature lowers the survival rate by as much as 50 p.c. This is not as great a drop in viability on thawing at room temperature as was noticed by Swim and colleagues (1958) with fibroblast lines, but it could well be a function of the type of cell under study.

Acknowledgment. I wish to thank Dr. P. L. Bazeley for his interest in this work.

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DETERMINATION OF PROTEIN FRACTIONS IN SMALL SAMPLES OF PLASMA AND OF LYMPH IN THE RABBIT

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(*Accepted for publication 7th July, 1960.*)

SUMMARY.

In an investigation of the transfer of proteins and lipids across the capillary membrane in the rabbit, only small volumes of lymph were often available. This necessitated the use of a method for determining the several protein fractions in lymph which involved the use of such small volumes. Total protein was determined by kjeldahl digestion and nesslerization, and the protein fractions by paper electrophoresis.

The various steps in the procedure of electrophoresis were standardized and the dye-binding capacities of the globulin fractions were determined in relation to albumin. All the globulin fractions were shown to bind less dye, bromophenol blue, than albumin and the areas of the dye curves were multiplied by the following factors in relation to albumin as unity: γ-globulin and fibrinogen, 1·76; β-globulin 1·40, α-globulin 1·50 and total globulin 1·56. The accuracy of the method for the determination of the levels of these fractions in the plasma and lymph of normal and hypercholesterolaemic rabbits is discussed.

INTRODUCTION.

In an investigation of the mechanisms concerned in the transfer of proteins and of lipids across the capillary membrane from plasma to lymph, it was necessary to determine the concentrations of the individual protein fractions in small samples of lymph collected from the lymphatic vessels of the liver and of the leg in the normal and hypercholesterolaemic rabbit (Courtice, 1960a; Courtice, 1960b). The procedure adopted was the determination of the total proteins by microkjeldahl digestion, and the separation and quantitative determination of the individual fractions by paper electrophoresis. For the kjeldahl determination 0·025 ml. and for paper electrophoresis 0·01 ml. plasma or lymph were used, so that duplicate determinations could be obtained for the different protein fractions on less than 0·1 ml.

The accuracy of measurement of the individual fractions by this method depends primarily on a clear separation of the proteins, but also on several other factors, such as the dye-binding capacity of the various fractions and the time and conditions of dyeing, staining and rinsing the paper strips (Jencks, Jetton and Durrum, 1955). In carrying out these measurements here, the latter factors

were standardized and under these conditions the dye-binding capacities of the different fractions were determined using the dye, bromophenol blue, for plasma of normal and hypercholesterolaemic rabbits. In this way it was hoped that a fairly accurate comparison of the protein fractions in plasma and lymph from these animals could be made.

Experimental Procedure and Results.

Plasma and lymph samples were obtained from normal and hypercholesterolaemic rabbits, and coagulation prevented by powdered heparin. Details of the procedure are described elsewhere (Courtice, 1959, 1960).

Total protein in plasma and lymph.

This was determined by kjeldahl digestion, distillation and nesslerization as follows: 0·025 ml. plasma, 1 ml. nitrogen-free H_2SO_4, 0·33 gm. K_2SO_4, 0·04 ml. 20 p.c. $CuSO_4$ and approximately 1 mg. selenium were introduced into a 25 ml. kjeldahl flask containing a glass head. This was boiled on a microkjeldahl electrothermal digestion apparatus with Simmerstat control for one hour after the contents became clear. It was found by trial of different times up to three hours that one hour was adequate for complete digestion of this amount of plasma. After cooling, the contents were transferred with the addition of 150 ml. distilled water to distillation flasks, and on the addition of 4 ml. 40 p.c. NaOH, the NH_4^+ was distilled by direct distillation in an all-glass still into 100 ml. graduated flasks and distillation continued until the level was about 3 ml. from the graduation mark. Two ml. of Nessler's solution were added, the level made up to the 100 ml. mark with water and the flask allowed to stand for 30 minutes before colour determination was made with a Beckman spectrophotometer at a wavelength of 420 mμ.

Throughout this procedure ammonia-free distilled water was used. This was prepared by passing distilled water through a column of zeo-carb 225 and testing the effluent with Nessler's solution.

The Nessler solution was made as follows: 45·5 gm. mercuric iodide and 36·6 gm. potassium iodide were dissolved in 200 ml. distilled water. When completely dissolved, 168 gm. potassium hydroxide dissolved separately in 500 ml. distilled water were poured in with shaking. Finally, a further 300 ml. distilled water were added, and the solution allowed to stand for 2-7 days before use.

(This method of kjeldahl digestion and nesslerization is, with modification to quantities, that used and supplied to me by Dr. A. C. Jennings of the Waite Institute, University of Adelaide, to whom I wish to express my thanks for his helpful advice.)

Non-protein nitrogen was similarly determined in the filtrate after precipitating the proteins of 0·5 ml. plasma in 4·5 ml. trichloracetic acid. As the level of N.P.N. is the same in lymph as in plasma (*cf.* Yoffey and Courtice, 1956), the figure for plasma was used in calculating the protein concentration of lymph from the total nitrogen value. The protein concentration was determined by assuming a nitrogen content of 16 p.c. for all fractions.

The accuracy of this method for measuring total protein was tested by determinations of known concentrations of pure bovine albumin. The results in Table 1 show that for the range of protein concentrations encountered in plasma and lymph, from 2 to 8 gm. per cent., the recovery is, on the average, 100 p.c. The scatter in such measurements was shown by making 20 determinations of 5 p.c. bovine albumin which gave a mean and standard error of the mean of $4·92 \pm 0·02$ gm./100 ml. and 20 determinations of an unknown plasma sample which gave a mean and standard error of the mean of $6·83 \pm 0·03$ gm./100 ml.

TABLE 1.

Determinations of known concentrations of pure bovine albumin.

Albumin concentration gm./100 ml.		
Actual	Determined	Percentage recovery
2·00	1·96	98
2·00	2·17	108
4·00	3·96	99
4·00	4·08	102
5·00	4·92	98
6·00	5·99	100
6·00	6·01	100
8·00	8·08	101
8·00	7·80	98

Determination of protein fractions.

Paper electrophoresis. This was carried out on Whatman No. 1 paper in a modified L.K.B. apparatus, using a barbiturate buffer of pH 8·6, at a voltage of 150V. for 16 hr. Four distinct fractions were always clearly separated, γ-globulin + fibrinogen, β-globulin, α-globulin and albumin. In some cases γ-globulin and fibrinogen could be clearly separated from one another as well as α_1 and α_2-globulins (*cf.* Morris and Courtice, 1955), but in the present measurements only the four fractions were estimated.

The strips were dried in an oven at 110° C. for 30 min. and stained with bromophenol blue (bromophenol blue 0·05 p.c., mercuric chloride 1 p.c., acetic acid 2 p.c. by volume) for 1¼ hr. The strips were then rinsed in 0·5 p.c. glacial acetic acid for four periods, one of 5 min. and three of 15 min. without agitation, and finally dried in the oven at 110° C. for 20 min.

The electrophoretic patterns were divided into consecutive sections 0·5 cm. wide, and each section eluted with a known volume (5 or 10 ml.) of 0·02N NaOH and the dye concentration determined with a Beckman spectrophotometer at a wavelength of 595 mμ. The figures were plotted, a curve drawn and vertical lines made from the separation of each of the main fractions, Fig. 1. The areas under the individual curves were then determined with a planimeter, so that the relative amounts of dye in each fraction could be measured.

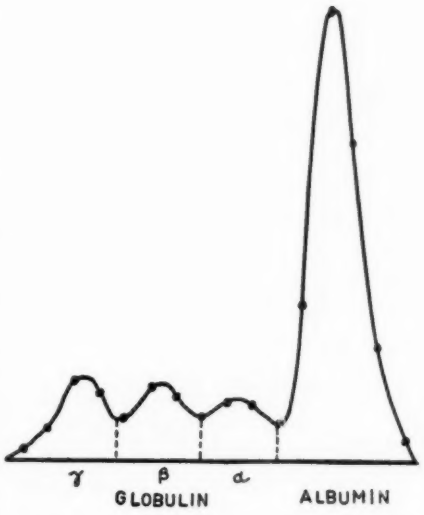

Fig. 1. A typical electrophoretic pattern showing the dye curves for the different protein fractions in rabbit plasma.

Dye-binding capacity of the protein fractions. The areas of the curves give a measure of the relative amounts of protein, only if the dye-binding capacity of 1 gm. of each fraction is the same. It has been clearly shown that for human serum the dye-binding capacity of the various proteins is not the same (*cf.* Lederer, 1957; Block, Durrum and Zweig, 1958). While some authors do not make any corrections, although recognizing this fact, others apply correction factors to the globulin fractions.

In these experiments the dye-binding capacity of the different fractions was determined by measuring the total nitrogen in one and the dye in the other of duplicate electrophoretic patterns run side by side on a 3-inch wide strip of paper, the same quantity of plasma, 0·03 ml., being used for each. After staining, the duplicate patterns were divided between each major fraction. For each fraction the dye was determined in one strip after elution with 0·02N NaOH and the protein was determined in the other by kjeldahl digestion using twice the quantities of digesting materials stated above. Control determinations of parts of the paper strip containing no protein showed only low blank readings, provided the barbiturate buffer was thoroughly removed by the normal rinsing after staining. Since the dye contains no nitrogen, the determinations of the total nitrogen in this way give an accurate measure of the amount of protein present in each fraction.

The results show that in rabbit, as in human plasma, a given amount of globulin binds less dye than the same amount of albumin. Therefore, the areas of the dye curves for the globulin fractions must be multiplied by a factor, X, to get an estimate of the true amount of protein present, according to the formula—

$$\frac{\text{X x dye in globulin fraction}}{\text{N}_2 \text{ in globulin fraction}} = \frac{\text{dye in albumin fraction}}{\text{N}_2 \text{ in albumin fraction}}.$$

The values for X for the various globulin fractions were determined in samples of plasma from 10 normal and 10 hypercholesterolaemic rabbits. The results are shown in Table 2. The differences in the two groups were not significant so the means for the 20 samples were used in calculating the relative amounts of protein. The areas of the dye curves were determined

TABLE 2.

The factors to be applied to the areas of the dye curves for the globulin fractions (albumin as unity) in calculating the concentrations of protein in these fractions in samples of rabbit plasma. Expressed as mean and standard error of mean.

Plasma	Total globulin	α-globulin	β-globulin	γ-globulin and fibrinogen
10 normal rabbits	1·60±0·05	1·58±0·12	1·46±0·06	1·74±0·05
10 hyper-cholesterolaemic rabbits	1·52±0·06	1·42±0·07	1·34±0·07	1·78±0·07
Total 20 rabbits	1·56±0·05	1·50±0·10	1·40±0·05	1·76±0·07

TABLE 3.

Values for plasma protein concentrations, gm./100 ml., in a group of 22 rabbits before and after applying correction factors for the different dye-binding capacities of proteins.

		Total protein	Albumin	α-globulin	β-globulin	γ-globulin and fibrinogen
Uncorrected	Mean	6·78	4·49	0·55	0·89	0·85
	S.E. of mean	±0·14	±0·13	±0·03	±0·05	±0·07
Corrected	Mean	6·78	3·80	0·69	1·05	1·25
	S.E. of mean	±0·14	±0·14	±0·03	±0·05	±0·10

as already described; then the γ-globulin fraction was multiplied by 1·76, the β-globulin by 1·40 and the α-globulin by 1·50. These corrected values gave the relative proportions of protein in each fraction and from the kjeldahl determination of the concentration of total protein in the sample, the concentrations of each fraction was calculated.

The effect of the corrections on the values obtained for the various fractions in samples of plasma from a group of 22 rabbits is shown in Table 3.

Accuracy of values for protein fractions.

The accuracy of this method of measurement of the various protein fractions depends, to a large extent, on the separation of the fractions on the paper. If the fractions are clearly separated the vertical lines in Fig. 1 can be fairly accurately drawn. The reproducibility of results was determined by using 10 strips with the same plasma on two different apparatuses. The mean and standard error of the mean for the different fractions were as follows in gm./100 ml.: albumin $3{\cdot}85 \pm 0{\cdot}03$, α-globulin $0{\cdot}59 \pm 0{\cdot}02$, β-globulin $1{\cdot}09 \pm 0{\cdot}02$ and γ-globulin and fibrinogen $1{\cdot}30 \pm 0{\cdot}02$.

In practice the measurements were made in duplicate and in Table 4 are given the results of 10 samples chosen at random.

Since lymph has a somewhat lower protein concentration than plasma, it was decided to test the accuracy of the method at these lower concentrations. This was done by determining the various fractions as a percentage of the total protein in whole plasma and in plasma diluted 1 in 3 and 2 in 3 with 0·9 p.c. sodium chloride. These dilutions would approximately represent the concentrations observed in hepatic and leg lymph respectively. The results obtained on samples of plasma from six rabbits with a fairly wide variation in the relative proportions of the protein fractions are shown in Table 5. It is clear that, within the limit of the errors already described, the same relative values are obtained when plasma is diluted considerably to resemble the concentrations observed in lymph.

TABLE 4.

Duplicate determinations of the protein fractions in ten samples of plasma or lymph taken at random expressed as gm./100 ml.

Total protein	Albumin	α-globulin	β-globulin	γ-globulin and fibrinogen
6·26	3·55	0·78	0·83	1·10
	3·53	0·73	0·87	1·13
6·93	3·68	0·86	0·99	1·41
	3·56	0·94	0·98	1·46
6·56	4·06	0·59	0·88	1·02
	4·10	0·64	0·91	0·91
5·38	2·42	0·75	1·19	1·02
	2·32	0·78	1·04	1·24
6·83	3·89	0·66	0·98	1·30
	3·83	0·68	1·01	1·31
7·38	4·85	0·69	0·94	0·90
	4·73	0·68	0·98	0·99
5·41	3·80	0·54	0·44	0·63
	3·80	0·44	0·49	0·68
7·50	4·92	0·84	0·87	0·87
	4·69	0·86	0·93	1·02
5·81	4·07	0·52	0·68	0·54
	3·88	0·65	0·74	0·54
5·44	3·64	0·41	0·68	0·71
	3·63	0·41	0·69	0·71

TABLE 5.

The total protein and the protein fractions expressed as a percentage of the total in samples of whole plasma[a] and of the same plasma diluted 1 in 3[b] and 2 in 3[c] with 0·9 per cent sodium chloride.

Rabbit No.	Sample	Total protein gm./100 ml.	Protein fractions as percentage of total			
			Albumin	α-globulin	β-globulin	γ-globulin and fibrinogen
1	a	7·41	56·1	8·6	10·0	25·3
	b	4·94	55·6	9·1	10·2	25·1
	c	2·47	53·7	10·0	10·1	26·2
2	a	6·83	56·4	8·6	16·0	19·1
	b	4·56	58·2	8·0	15·7	18·1
	c	2·28	59·2	8·9	14·0	17·9
3	a	7·50	64·0	11·3	12·0	12·7
	b	5·00	64·7	11·7	10·6	13·0
	c	2·50	64·7	10·7	11·1	13·5
4	a	7·38	65·0	9·2	13·0	12·8
	b	4·92	63·9	9·9	13·5	12·7
	c	2·46	65·3	9·7	12·3	12·8
5	a	7·40	65·9	8·0	13·5	12·6
	b	4·94	62·6	9·5	13·7	14·1
	c	2·47	64·5	10·5	12·5	12·5
6	a	5·41	70·2	9·0	8·6	12·2
	b	3·60	69·4	9·6	8·9	12·2
	c	1·80	69·3	10·3	8·6	11·8
Mean	a	6·99	62·9	9·1	12·2	15·8
	b	4·66	62·4	9·6	12·1	15·9
	c	2·33	62·8	10·0	11·4	15·8

Proteins in hepatic and leg lymph.

The proteins in hepatic and leg lymph in a group of 15 rabbits are shown in Table 6.

The significance of the composition of hepatic and leg lymph with regard to the permeability of the capillaries in those regions will be presented elsewhere in greater detail (Courtice, 1960a; 1960b).

TABLE 6.

Concentrations of total protein and of the various fractions, gm./100 ml., of plasma, hepatic lymph and leg lymph in a group of 15 rabbits. Expressed as mean and standard error of the mean.

	Total protein	Albumin	α-globulin	β-globulin	γ-globulin and fibrinogen
Plasma	6·69 ±0·17	3·58 ±0·14	0·71 ±0·04	1·10 ±0·06	1·31 ±0·12
Hepatic lymph	5·46 ±0·21	3·24 ±0·17	0·51 ±0·03	0·76 ±0·04	0·94 ±0·10
Leg lymph	2·79 ±0·19	1·70 ±0·15	0·27 ±0·02	0·37 ±0·03	0·44 ±0·05

DISCUSSION.

Although the quantitative determination of the protein fractions in plasma and lymph by paper electrophoresis is tedious and open to certain criticisms, it is the only method that can be used for small samples of lymph. Provided the procedure is standardized, one of the main difficulties is the different dye-binding capacities of the proteins. Because of the variation in dye-binding capacity, some authors have not applied corrections (Flynn and de Mayo, 1951; Grassman, Hannig and Knedel, 1951; Sommerfelt, 1952; Kellner, 1954). Cremer and Tiselius (1950) and Kunkel and Tiselius (1951) multiplied the dye in the globulin fraction by 1·6. Esser, Heinzler, Kazmeier and Scholtan (1952), taking albumin as unity, multiplied the dye values for α_1-globulin by 2·36, α_2-globulin by 2·09, β-globulin by 2·20 and γ-globulin by 1·62. Koiw, Wallenius and Gronwall (1952) used correction factors of 2·8 for α_1-globulin, 1·7 for α_2-globulin, 1·6 for β-globulin, 1·4 for γ-globulin, and 1·6 for total globulin in normal human plasma, but they found that in various diseases, the dye-binding capacity of the various proteins varied considerably. Most of these investigations have been made on human serum for use in clinical pathology.

In the present series of experiments on plasma samples from normal and hypercholesterolaemic rabbits, the dye-binding capacity of each globulin fraction in relation to that of albumin was fairly constant. Although such problems as "tailing" of the albumin fraction were not taken into consideration, it seemed justifiable to apply the corrections to the dye curves obtained from the electrophoretic patterns of plasma and lymph samples in the rabbit, especially in determining the plasma : lymph gradients of the different fractions in the same animal, which was the purpose of the investigation.

It is apparent from the different results in the literature that, before this method is used for a specific purpose, the dye-binding capacities of the different fractions should be determined for the type of paper and of dye used, the animal species, the state of the animal and the conditions of electrophoresis. If this is done, many, but not all, of the criticisms are removed, and the protein fractions can be determined quantitatively in small samples of plasma or lymph with the degrees of accuracy obtained in these experiments. It should be realized, however, that the globulin fractions so measured do not each represent a homogeneous protein, but a mixture of many proteins which may have different chemical and physical properties, but approximately the same electrophoretic mobility.

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THE FLOW AND COMPOSITION OF HEPATIC LYMPH IN THE NORMAL AND HYPERCHOLESTEROLAEMIC RABBIT

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(*Accepted for publication 7th July, 1960.*)

SUMMARY.

Hepatic lymph was collected from an efferent vessel of the hepatic lymph node in two groups of rabbits, one living on a normal diet and one on a diet to which cholesterol was added.

The average flow of lymph in the normal group was 0·7 ml./hr., and much greater, up to 30 ml./hr., in the hypercholesterolaemic animals.

The average albumin level in the lymph of the normal rabbit was 96 p.c. and the globulin 74 p.c. of that in the plasma. In the hypercholesterolaemic group these values were slightly less, 88 and 70 p.c. respectively. Each of the globulin fractions, α, β and γ, varied only slightly from the mean globulin level.

The cholesterol and phospholipid plasma : lymph gradients in the normal animals were much the same as for α and β globulin, but in the hypercholesterolaemic group these gradients were much greater. This was in agreement with the findings that the increased lipid levels in the plasma of the hypercholesterolaemic rabbit were due mainly to larger, less dense complexes with a high cholesterol : phospholipid ratio.

The significance of these findings with regard to the permeability of the hepatic sinusoids and the production of lymph is discussed.

INTRODUCTION.

In the liver the lymphatic capillaries form a network at the periphery of each lobule. It is thought that tissue fluid in the Dissé space, between the sinusoidal membrane and the liver cells, drains into these vessels to form the lymph that is ultimately carried away by the hepatic lymph ducts emerging from the hilus (cf. Yoffey and Courtice, 1956). This tissue fluid, as in other regions of the body, will come primarily from the plasma by filtration through the sinusoidal membrane. Since the membrane is very permeable to protein and the hepatic tissue fluid pool is small (Morris, 1956a), there is a rapid turnover. The composition of the capillary filtrate may also be modified by exchanges of substances with the liver parenchymal cells; this may be especially so with regard to protein and certain lipids (cf. Frederickson and Gordon, 1958).

Although the sinusoidal membrane has been shown by electron microscopy to be either absent in part or to contain such large gaps between the endo-

thelial cells as to preclude molecular sieving of albumin and globulin, there is nevertheless a different plasma : lymph gradient for albumin than for globulin (Morris, 1956b). In the present series of experiments it was decided to extend these observations to a much larger range of different sized molecules or complexes. It was hoped that in the rabbit that had been maintained hypercholesterolaemic for many weeks, the lipid complexes, varying in size from 150Å to 2000Å, would be in a "steady state" during lymph collection. An investigation of the plasma : lymph gradients of albumin, globulin and the different lipoprotein molecules or complexes should in the circumstances be useful in a study of the permeability of the sinusoidal membrane to macromolecules of this size.

Methods.

General procedure. Hutch-bred rabbits of various strains were used. Hypercholesterolaemia was induced by feeding a diet to which cholesterol was added (Courtice, 1959a). The animals were anaesthetized with nembutal for the cannulation of the hepatic lymphatics and the collection of lymph.

Hepatic lymph was collected usually from an efferent lymphatic duct as it coursed over the pancreas after emerging from the main hepatic lymph node which lies adjacent to the portal vein near the porta hepatis. In a few cases lymph from an afferent vessel to this node was collected. These lymphatic ducts in the rabbit are in a somewhat similar position to those in the dog and cat, but are much more thin-walled and fragile and so more easily torn during cannulation. Cannulation was relatively easy in the hypercholesterolaemic animals in which the lymph flow was much greater than in the normal. In most cases, about 1 cm. of the end of a 27-gauge needle, to which transflex tubing was attached, was used as a catheter and inserted directly into the lymphatic vessel; in others, transflex tubing was inserted after incision of the vessel, while in a few normal animals where cannulation failed, lymph was collected by a Pasteur pipette as it issued from the incised lymph duct.

Lymph was collected over a period of one hour as one or as several samples depending on the flow. A blood sample was obtained during lymph collection from a cannula in the carotid artery. In both lymph and blood samples, clotting was prevented by powdered heparin.

Analytical methods. Total protein and the protein fractions were determined in plasma and lymph as described by Courtice (1960a). Total cholesterol was determined by the method of Abell, Levy, Brodie and Kendall (1952) and phospholipid by the method of Zilversmit and Davis (1950). The plasma lipids were separated by a Spinco model L preparative ultracentrifuge into three fractions containing lipids of the following densities, $< 1{\cdot}019$, $1{\cdot}019$-$1{\cdot}063$ and $> 1{\cdot}063$, according to the method of Havel, Eder and Bragdon (1955). The fraction of density $> 1{\cdot}063$ will be referred to as α-lipoprotein, and that of density $1{\cdot}019$-$1{\cdot}063$ as β-lipoprotein, although it is realized that each of these fractions may not contain a single homogeneous lipoprotein.

RESULTS.

Lymph flow from the liver.

In these experiments it was not possible to cannulate a single duct which drained all the lymph emerging from the hilus of the liver. In the dog and

cat, most of the hepatic lymph may be collected in this way, but in the rabbit there were as a rule two or three vessels emerging from the hepatic lymph node. These were ligated and one cannulated. The lymph flow was measured over a period of 30 to 60 minutes in a group of normal and a group of hypercholesterolaemic animals. The results are given in Table 1. Although these figures

TABLE 1.

Lymph flow from the liver in normal and hypercholesterolaemic rabbits.

Normal			Hypercholesterolaemic			
Rabbit No.	Wt. Kg.	Lymph flow ml./hr.	Rabbit No.	Wt. Kg.	Duration of hypercholesterolaemic weeks	Lymph flow ml./hr.
40	3·3	0·6	50	2·2	1	2·0
46	2·1	0·3	49	1·6	2	1·5
58	2·8	0·8	51	3·2	2	0·4
60	2·5	1·0	48	2·0	4	2·0
63	2·6	0·4	20	2·3	8	1·7
64	2·5	0·8	21	2·1	8	1·5
65	2·5	0·8	30	2·2	11	27·0
Mean	2·5	0·7	31	2·4	11	2·4
			32	2·4	11	30·0
			33	2·5	11	12·0
			41	2·8	12	9·0
			43	2·4	12	10·0
			44	2·0	12	24·0
			47	2·2	13	9·0

may not give an accurate measure of the total lymph flow from the liver, they do show the large difference between the normal and hypercholesterolaemic animals. In the normal animal the lymphatic vessels were not very evident and the lymph flow was usually less than 1 ml. per hour. In those animals that were hypercholesterolaemic for only a few weeks, the flow was much the same or a little higher, but in those of three months' duration the lymphatics emerging from the hilus were widely dilated and the flow from these vessels was very high, up to 30 ml. per hour. In none of the hypercholesterolaemic animals was the lymph flow less than in a normal animal indicating that in these experiments the hepatic lymphatics were never blocked by deposits of cholesterol. The increase in lymph flow was probably due to cirrhosis of the liver resulting from hypercholesterolaemia (Leary, 1941), and followed the same course as in cirrhosis caused by other agents (cf. Nix, Flock and Bollman, 1951; Nix, Mann, Bollman, Grindlay and Flock, 1951).

The lipids in the plasma of the normal and hypercholesterolaemic rabbit.

In the rabbit fed on a normal diet the lipids in the plasma are low in concentration and present largely in the high density α-lipoprotein with a low cholesterol : phospholipid ratio (Morris and Courtice, 1955). When cholesterol is added to the diet, the plasma lipids increase and the cholesterol : phospholipid ratio increases. In the present experiments these changes are depicted in Fig. 1.

With rising concentrations of cholesterol in the plasma, the lipids are present mainly in a fraction less dense than β-lipoprotein (cf. Gofman, Lindgren, Elliott, Mantz, Hewitt, Strisower and Herring, 1950). In the present series of experiments, the lipids were separated into three fractions as described and the mean values for four normal and nine hypercholesterolaemic animals are given in Fig. 2. It is clear that there was little increase in the high density fraction (α-lipoprotein), an appreciable increase in the fraction of density 1·019-1·063 (β-lipoprotein) and very considerable increase in the low density fraction, $< 1{\cdot}019$. The cholesterol : phospholipid ratio increased greatly in the two less dense fractions. These changes are described in more detail elsewhere (Courtice and Garlick, 1960).

The size of the lipid complexes in these fractions is being investigated with the electron microscope and preliminary investigations suggest that they vary in size from 150A to 1500A with some larger ones. The complexes in the frac-

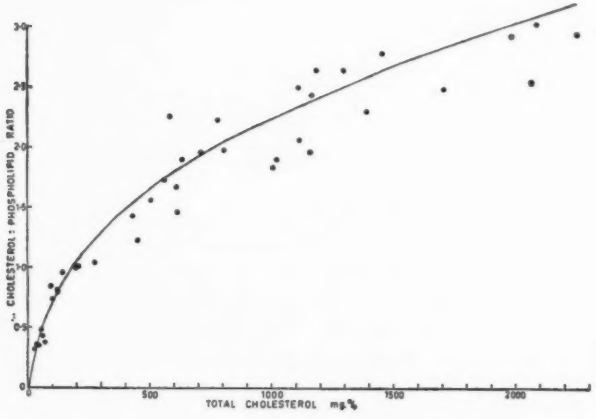

Fig. 1. The relation between the level of total cholesterol in mg./100 ml. and the cholesterol : phospholipid ratio in the plasma of normal and hypercholesterolaemic rabbits.

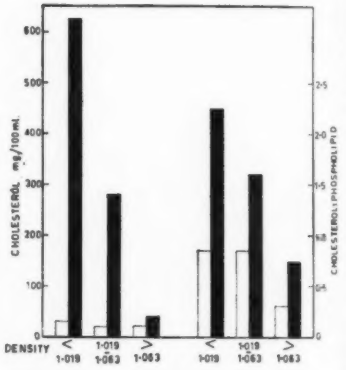

Fig. 2. The cholesterol expressed in mg./100 ml. plasma, and the cholesterol : phospholipid ratio in the three fractions of densities $< 1{\cdot}019$, $1{\cdot}019$-$1{\cdot}063$ and $> 1{\cdot}063$, in plasma of the normal and hypercholesterolaemic rabbit.
□ Mean of 4 normal animals.
■ Mean of 9 hypercholesterolaemic animals.

tion of lowest density are the largest and are responsible for the milky appearance of the plasma of the hypercholesterolaemic rabbit. In hypercholesterolaemia, therefore, the proteins and lipids in the plasma are present as molecules or complexes which vary in size from albumin with a diameter of 38Å to the largest lipoprotein complexes of 1500Å to 2000Å.

The composition of hepatic lymph in relation to plasma.

The protein and lipid concentrations of plasma and lymph in groups of 11 normal and 19 hypercholesterolaemic rabbits are shown in Table 2. The individual fractions were determined in seven normal and in nine hypercholesterolaemic animals.

The different proteins and lipids in the lymph are presented in Fig. 3 as percentages of the levels in the plasma. It is evident that in both the normal and the hypercholesterolaemic groups, the relative concentration of albumin in the lymph was greater than that of globulin as a whole and of each of the individual globulin fractions. In the normal animals, the albumin level was on an average 96 p.c., whereas that of the globulins was 74 p.c. of the concentrations in the plasma. This difference may be due to different diffusion rates of the molecules because of their size or it may be due to the production of new albumin by the liver cells. In the hypercholesterolaemic animals, the values were slightly lower, but a similar difference between albumin and globulin

TABLE 2.

The protein and lipid levels in the plasma and hepatic lymph of normal and of hypercholesterolaemic rabbits, expressed as the mean and standard error of the mean.

	Total protein g/100 ml.	Albumin g/100 ml.	α-globulin g/100 ml.	β-globulin g/100 ml.	γ-globulin and fibrinogen g/100 ml.	Cholesterol mg./100 ml.	Phospholipid mg./100 ml.
Normals (11)							
Plasma	6·86	–	–	–	–	130	153
	±0·14					± 18	± 13
Lymph	5·80	–	–	–	–	79	109
	±0·15					± 11	± 6
Normals (7)							
Plasma	6·76	3·69	0·72	1·01	1·33	138	154
	±0·18	±0·11	±0·05	±0·08	±0·17	± 22	± 16
Lymph	5·82	3·56	0·54	0·81	0·90	85	111
	±0·20	±0·13	±0·04	±0·08	±0·13	± 13	± 7
Hypercholesterolaemic (19)							
Plasma	6·73	–	–	–	–	1153	511
	±0·16					±143	± 37
Lymph	5·54	–	–	–	–	430	242
	±0·21					± 71	± 24
Hypercholesterolaemic (9)							
Plasma	6·66	3·49	0·70	1·19	1·28	798	407
	±0·25	±0·23	±0·06	±0·08	±0·17	±117	± 36
Lymph	5·28	3·06	0·49	0·77	0·97	274	191
	±0·33	±0·24	±0·04	±0·07	±0·13	± 48	± 30

was evident. The slightly lower values for the protein fractions in lymph in the hypercholesterolaemic animal may be due to the higher filtration rate giving rise to a higher lymph flow, or to the liver being less efficient in forming these fractions. The fact that there was no appreciable change from the normal in the levels of proteins in the plasma of the two groups supports the former view.

When the level of protein in the lymph was plotted against that in the plasma, Fig. 4, it was evident that the slope of the regression line for albumin was steeper than that for globulin. These regression equations also showed

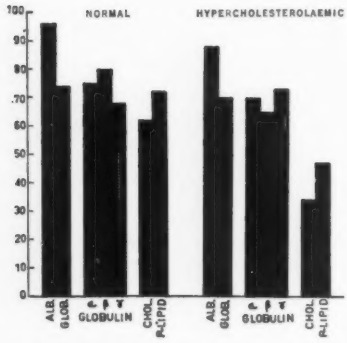

Fig. 3. The levels of albumin, total globulin, α, β and γ globulins, cholesterol and phospholipid in hepatic lymph expressed as a percentage of the corresponding plasma level. Mean figures for 7 normal and 9 hypercholesterolaemic animals.

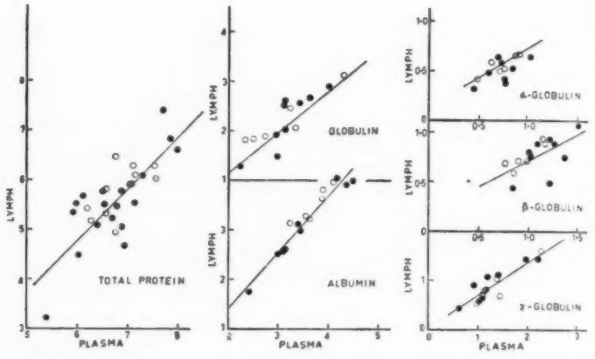

Fig. 4. The levels of protein in the lymph plotted against that in plasma, expressed as g./100 ml. The lines were drawn from the determined regression equations.
○ Normal.
● Hypercholesterolaemic.

that the levels of the proteins in the lymph were directly related to those in the plasma.

The mean cholesterol level in the lymph of the normal animals was 62 p.c. of the plasma level. This was slightly less than the relative levels of α and β globulins indicating a small differential in the filtration of the lipoproteins and of the corresponding globulins. In hypercholesterolaemia, however, where the cholesterol was present mainly in much larger complexes, the level in the lymph was only 34 p.c. of that in the plasma. These results suggest that there was a relatively greater filtration of the smaller molecules with a lower cholesterol : phospholipid ratio.

This was shown also when the level of cholesterol in the lymph was plotted against that in the plasma, Fig. 5. For low plasma levels there was a greater percentage of cholesterol in the lymph, than at higher levels, although this was much less marked in the liver than in the limb (cf. Courtice, 1960b).

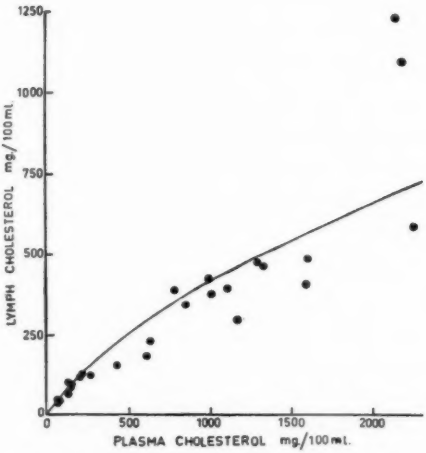

Fig. 5. The level of cholesterol in the hepatic lymph plotted against that in the plasma in normal and hypercholesterolaemic rabbits.

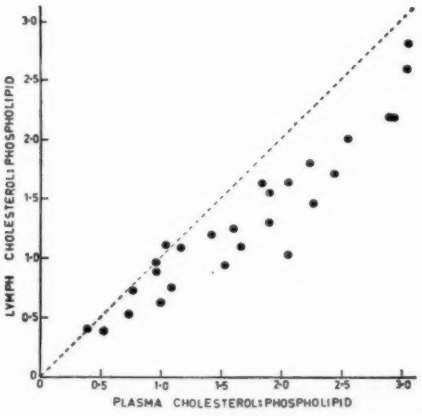

Fig. 6. The cholesterol : phospholipid ratio in the hepatic lymph plotted against that in the plasma of normal and hypercholesterolaemic rabbits.

The same conclusions could be drawn when the cholesterol : phospholipid ratio of the lymph was plotted against that of the plasma, Fig. 6. The interrupted line indicates the theoretical regression line when the ratio in the lymph is the same as in the plasma. At low plasma levels, where the lipids were present as relatively small molecules, the values approximated to this line, but at higher levels there was a deviation from the line indicating that diffusion of the larger complexes was restricted more than that of the smaller ones.

DISCUSSION.

Lymph flow.

The average hilar lymph flow in a group of normal rabbits was 0·7 ml./hr. or about 0·3 ml./kg./hr. This is somewhat less than the flow of 0·73 ml./kg./hr. in the cat (Morris, 1956b), and 0·66 ml./kg./hr. in the dog (Ritchie, Grindlay and Bollman, 1959). It is likely, however, that the actual figure in the rabbit may be higher since the hilar lymph did not always pass into one common efferent trunk.

The origin of this lymph is probably from the fluid in the Dissé space between the parenchymal cells and the sinusoidal wall. Electron microscopy has shown that this edge of the parenchymal cells consists of villiform processes. Fawcett (1955) showed that in places there seemed to be an absence of the

sinusoidal membrane, while Bennett, Luft and Hampton (1959) described large gaps between the endothelial cells. These gaps were several thousands Å in diameter, and would, therefore, offer little restriction to diffusion, even of the larger complexes described here.

In the hypercholesterolaemic animals, the hepatic lymph flow was much greater than in the normal, especially in those of 8 to 12 weeks' duration in which cirrhosis was present (cf. Leary, 1941). In these animals, the plasma and lymph were sometimes deeply bile-stained and at *post mortem* the liver showed, both macroscopically and microscopically, the cirrhotic changes described by Leary. The lymphatic vessels emerging from the hilus of the liver were grossly distended, similarly to those in cirrhosis in man (Baggenstoss and Cain, 1957) and experimental cirrhosis in the dog and rat (Nix *et al.*, 1951). The greater lymph flow in these conditions is probably due to an increased intrahepatic portal pressure leading to an increased filtration and turnover of tissue fluid in the liver (cf. Courtice, 1959b).

Composition of hepatic lymph.

The plasma : lymph gradients of albumin and globulin are difficult to explain on the morphological structure observed by electron microscopy. There should be no appreciable molecular sieving of molecules of the size of albumin and globulin to account for the findings that in the normal group the albumin in the lymph was 96 p.c. and the globulin only 74 p.c. of the plasma levels. The cholesterol in the lymph was 62 p.c. and phospholipid 72 p.c., slightly less than the total globulin; these figures are in agreement with the finding that in the normal rabbit most of the cholesterol is in the form of α-lipoprotein. Morris (1956a) also found a difference in the plasma : hepatic lymph gradients of albumin and globulin in the cat with the cholesterol gradient about the same as for globulin, but the difference was less marked.

The same differences were observed in the group of hypercholesterolaemic rabbits, where the albumin in the lymph was 88 p.c. and globulin 70 p.c. of the plasma levels. In this group, however, the lymph cholesterol was only 34 p.c. and the phospholipid 47 p.c. of the plasma levels.

The specific activities of these various molecules and complexes have not yet been determined, so it is not possible to say for certain that these substances in the lymph were derived only by filtration of the plasma. The experiments of Morris (1956b) in the cat, however, do suggest that the albumin in the lymph is mainly derived in this way.

It is probable that there is an exchange of the lipids between the plasma and liver cells. In these experiments where there was no change in the plasma levels during the course of the experiment, and where the animal had been in a "steady state" with regard to the plasma lipids for a considerable time, it seems probable that an equilibrium was reached between the lipids in the tissue fluid and in the parenchymal cell. In all cases the animals had not received choles-

terol in the diet for 2 to 14 days. In such circumstances the lymph should reflect the average composition of the capillary filtrate as far as these substances are concerned. The differences in the plasma : lymph gradients, therefore, would suggest that there must be molecular sieving of the different sized molecules and complexes. Such a phenomenon would not occur if all the gaps between the endothelial cells were several thousand Å across. It seems probable, therefore, that the structure of the sinusoidal membrane varies and that while in some parts there is a fairly free interchange of plasma and tissue fluid, in others there may be considerable molecular sieving of all molecules of the size of albumin and greater.

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THE EFFECT OF INCREASED MUSCLE BLOOD FLOW ON EXERCISE HYPERAEMIA IN THE HUMAN FOREARM

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(*Accepted for publication 7th July, 1960.*)

SUMMARY.

Passive raising of the legs and trunk elevated forearm blood flow, before, during and after rhythmic and sub-maximal sustained contraction of human forearm muscle.

The magnitude and rate of decline of post-exercise hyperaemia in the forearm was unaffected by the increase in muscle blood flow produced by passive raising of the legs and trunk.

This finding suggests that either the metabolites responsible for post-exercise hyperaemia are removed by a mechanism independent of the total volume of blood flowing, or that passive raising of the legs and trunk increases blood flow through channels not concerned with the transfer of metabolites formed during exercise.

INTRODUCTION.

The hyperaemia that occurs in a muscle during and following contraction has been attributed to the accumulation of vasodilator metabolites (Gaskell, 1877; Anrep and Von Saalfeld, 1935). The nature of these substances is still unknown.

If the rate of removal of the metabolites is related to the rate of blood flow, then increasing flow during and following exercise might be expected to diminish the degree of hyperaemia. Barcoft *et al.* (1952) attempted to do this by the technique of indirect heating, and found that although the forearm blood flow was elevated considerably by this procedure it did not influence the hyperaemia following forearm muscle exercise. It has subsequently been shown, however, that indirect heating causes reflex dilatation of skin blood vessels only, the muscle vessels being unaffected (Roddie *et al.*, 1956). Dornhorst and Whelan (1953) and Patterson and Shepherd (1954) increased total forearm blood flow by administration of adrenaline, adenosine triphosphate, histamine and acetylcholine, and found no change in the magnitude and rate of decline of post-exercise hyperaemia. There was no direct evidence, however, that these drugs

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were increasing muscle blood flow, although it seemed likely that this was the case. In addition, adrenaline, by increasing muscle metabolism, might itself produce the same dilator metabolites.

Passive elevation of the legs and trunk has recently been shown to cause a reflex dilatation in the forearm which is confined to the muscle vessels alone (Roddie and Shepherd, 1956; Roddie *et al.*, 1957). This technique has been used to re-examine the influence of raised muscle blood flow on post-exercise hyperaemia.

METHODS.

The subjects were healthy young adult males who lay for half an hour at rest on a couch prior to the first recordings. Room temperature was kept constant at 22° C. ± 1°.

Forearm blood flow was measured by venous occlusion plethysmography using water-filled plethysmographs at a temperature of 35° C. (Greenfield, 1954).

The standard exercise consisted of raising a column of water or mercury in a 1 cm. diameter glass tube by squeezing a rubber bulb either as a sub-maximal sustained contraction or rhythmically to a metronome at a rate of 30 contractions per minute. Actual work done varied slightly between experiments but was constant for the particular subject.

Muscle blood flow was increased by passive raising of the legs and trunk (Roddie and Shepherd, 1956; and Roddie *et al.*, 1957). This was effected rapidly and comfortably by ropes and pulleys attaching the feet to an overhead scaffolding.

During sustained contraction blood flow was measured in the normal manner, but during rhythmic contraction the method of Barcroft and Dornhorst (1949) was employed.

Following exercise, blood flow was recorded for six minutes with particular attention to the first two minutes to ensure accurate peak flow readings (Patterson and Whelan, 1955). A ten-minute recovery period separated consecutive runs, and blood flow frequently remained slightly elevated above the initial resting level even after this time.

Blood flow was plotted against time to the nearest 5 sec. on graph paper and the total volume of excess blood flowing above the post-exercise resting level was determined by weighing the area under the curve following exercise. In this way the size of the hyperaemias could be compared between the horizontal and legs raised position. The rates of decrease of the hyperaemias were compared by plotting the logarithm of flow rate against time during the first minute following exercise.

RESULTS.

Leg and trunk raising increased forearm flow in five of seven subjects tested. Only experiments in which resting flow was increased by at least 2 ml./100 ml. of forearm tissue/min. are included and this corresponds to an increase of approximately 10 ml./min. or more through muscle at rest. The greatest single increase recorded was 42 ml./min.

Reproducibility of the size and rate of decline of the hyperaemia in response to standard exercise was checked in each experiment by comparing hyperaemias from repeated exercise with the body horizontal. In this way the total volume of blood flowing was found to be reproducible to within 3 ml. and the range of volume varied from 11 to 70 ml. in different subjects depending on the work done.

Forearm blood flow during exercise.

Rhythmic contraction. Thirteen observations on two subjects revealed that leg raising consistently increased blood flow during exercise. On the average this amounted to an increase from 6·1 to 9·4 ml./100 ml./min. in one subject and 9·4 to 11·7 ml./100 ml./min. in the other. Fig. 1 illustrates the changes

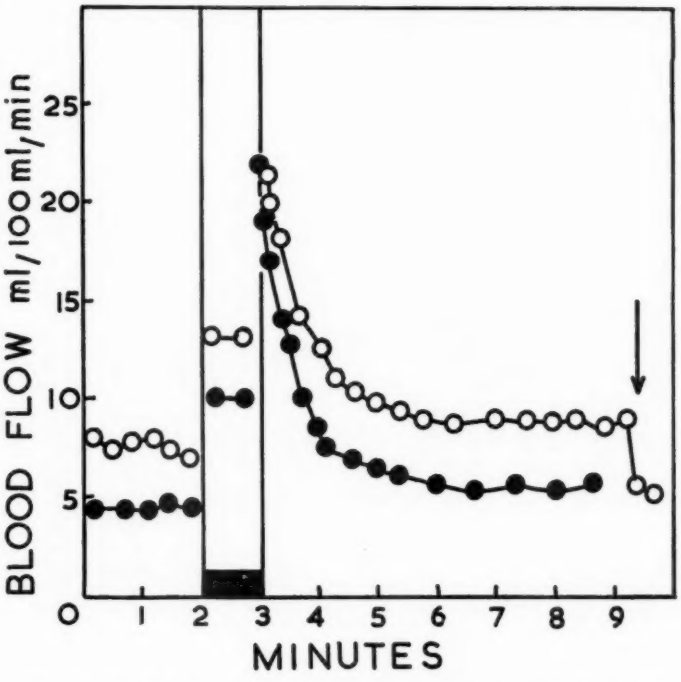

Fig. 1. *Blood flow (in ml./100 ml. of forearm tissue/min.) in one subject before, during and after rhythmic contraction of the forearm muscles for one minute (black rectangle).* Closed circles body horizontal. Open circles legs and trunk raised. At the arrow the body was returned to the horizontal.

in blood flow that resulted from leg and trunk raising in one experiment before, during and after rhythmic exercise. The resting flow was elevated by approximately 3 ml/100 ml./min. and this was maintained during and following the period at rhythmic contraction. The body was returned to the horizontal position at the arrow and forearm blood flow immediately reverted to the control level.

Sustained contraction. Nine observations on two subjects showed that on the average blood flow was increased during sub-maximal sustained contraction from 5·7 to 6·8 ml./100 ml./min. in one subject and 7·9 to 11·3 ml./100 ml./min.

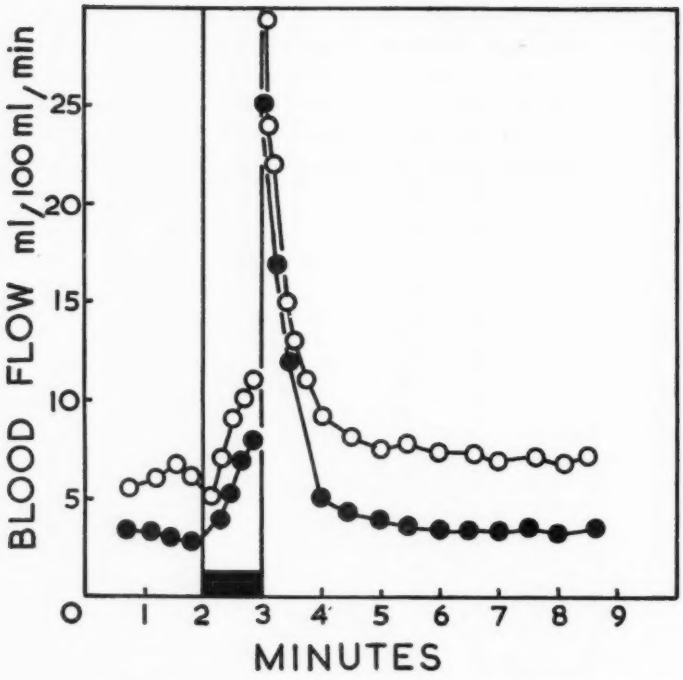

Fig. 2. *Blood flow (in ml./100 ml. of forearm tissue/min.) in one subject before, during and after sub-maximal sustained contraction of the forearm muscles for one minute (black rectangle).* Closed circles body horizontal. Open circles legs and trunk raised.

in the other. Fig. 2 illustrates the change in one experiment during sub-maximal sustained contraction. Following leg raising the resting flow was elevated by approximately 2·5 ml./100 ml./min. and this was largely maintained throughout the period of exercise.

Post-exercise hyperaemia with the legs raised.

Twenty-six paired observations on five subjects during rhythmic contraction and eight paired observations on two subjects during sustained contraction showed no significant differences in total volume of excess blood flowing above post-exercise resting level between the horizontal and the legs raised position (Table 1). Figs. 1 and 2 show that leg raising simply elevated the position of the hyperaemia on the graph such that the area under the curve related to the post-exercise resting level was unaltered.

TABLE 1.

Total volume of blood flowing (ml) above the post-exercise resting level following forearm exercise with legs horizontal and elevated.

Observations		D Legs down	U Legs up	Difference D−U
(a) Rhythmic contraction	1	19·3	16·4	+ 2·9
	2	17·9	13·8	+ 4·1
	3	38·6	39·5	− 0·9
	4	23·4	28·9	− 5·5
	5	24·0	18·9	+ 5·1
	6	23·2	15·9	+ 7·3
	7	32·8	23·0	+ 9·8
	8	15·4	15·9	− 0·5
	9	14·0	10·6	+ 3·4
	10	13·7	12·3	+ 1·4
	11	12·2	10·2	+ 2·0
	12	12·0	15·9	− 0·5
	13	11·7	14·7	− 3·0
(b) Sustained contraction	1	23·3	32·2	− 8·9
	2	25·6	20·8	+ 4·8
	3	60·0	49·1	+10·9
	4	58·3	73·8	−15·5

There is no significant change in volume of excess blood flowing after exercise of the forearm muscles when the legs and trunk are raised from the horizontal. For rhythmic contraction $P > 0{\cdot}1$.

A source of error in this technique is seen in Fig. 1 where the first high blood flow following exercise with the legs and trunk raised has been missed. This type of error is one cause of the small discrepancy in the reproducibility of these hyperaemias.

No consistent difference was observed in the rate of decrease of blood flow in the first minute following exercise.

DISCUSSION.

If exercise hyperaemia is produced by release of a dilator metabolite which is highly diffusible such that its rate of removal depends upon and actually regulates blood flow, then a significant reduction in hyperaemia would be expected to result from procedures that increase muscle blood flow while not affecting its metabolism.

Leg raising causes large increases in muscle blood flow before, during and after contraction of the forearm muscles and yet produces no effect on the magnitude of post-exercise hyperaemia. There are two possible explanations for this finding:

Firstly, that if dilator metabolites are responsible for post-exercise hyperaemia then they have a limiting rate of diffusion such that their removal is independent of the volume of blood flowing, so long as it is above some minimum critical level. This was first suggested by the experiments of Anrep

and Von Saalfeld (1935) and is in accord with the conclusions of Dornhorst and Whelan (1953) and Patterson and Shepherd (1954). Furthermore, it is compatible with the findings of Blair *et al.* (1959) who showed by graded compression of the brachial artery that the excess blood flowing during the hyperaemic period was not necessary for removal of the vasodilator metabolites.

Secondly, that the increase in muscle blood flow produced by leg and trunk raising does not occur through channels participating in the transfer of metabolites. Morphological evidence for arterio-venous shunts in skeletal muscle has been obtained by Zweifach and Metz (1955), Zweifach (1957), Saunders *et al.* (1957), and Griffin (1959), and convincing indirect evidence by Hyman (1957), Hyman *et al.* (1959) and Barlow *et al.* (1959). Hyman (1960) reported that tissue clearance of Na^{24} was unaffected by leg raising and suggested that the muscle dilatation may be attributed to the opening of arterio-venous anastomoses. If this were the case the excess blood flowing after exercise might be unchanged by leg raising regardless of the diffusion properties of vasodilator metabolites.

It is not possible at present to decide which of these two explanations is the more likely.

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BLOOD pH DETERMINATION

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(*Accepted for publication 14th July, 1960.*)

SUMMARY.

The paper describes a simple and inexpensive apparatus for the measurement of pH at 38° using only 0·15 to 0·2 ml. of blood. The electrode bulb nearly fills the bore of the electrode vessel. There is a wick junction but no tap between the 3·5 M KCl calomel half-cell and the electrode vessel. This last is filled and emptied with bent capillary pipettes. The bath contains kerosene heated with a bare coil which receives 2 amp. at 12 volts. It is controlled with a small mercury-in-glass regulator and a relay.

INTRODUCTION.

The interest taken in blood electrolyte studies has led to the appearance of numerous pieces of apparatus for the determination of blood pH. From a survey of the literature it would seem that great precision is usually expected. Much of the equipment is fairly expensive, suitable for large wealthy institutions with adequate highly trained staffs. In some instruments mercury is used to put the blood in the electrode vessel without allowing much air to enter; this constitutes a serious hazard to the health of the workers. Others, e.g. Behrmann and Fay (1939), have a degree of temperature control which is inadequate for the accuracy that is often desired. Some, more complex, use one of the somewhat costly water-circulating systems, of which the long hoses impair the temperature regulation. Even at "laboratory temperatures" a constant temperature bath is essential for good work. Again, e.g. Sanz (1957), essential details of the construction are not always revealed. Equipment is often far from easy to clean, it may include costly proprietary electrode systems that are available only in some places and the amount of fluid necessary for a determination is frequently inconveniently large.

The design of the apparatus to be described was based on the following considerations: the amount of fluid used must not exceed 0·2 ml. or 0·15 ml. for a careful worker; blood and other proteinous fluids rapidly impair small electrodes (Guthe, 1959); taps, especially ungreased glass ones, are apt to stick and be broken; the whole apparatus had to be made and operated at the least possible cost and without assistance from highly-trained laboratory craftsmen. It is, accordingly, simple, cheap and easily made. It is dismantled in a few minutes

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

for cleaning; it can be readily laid aside when not required and as readily brought into action when needed.

Method and Materials.

Though the glass electrode system that is here described can be used with any pH meter the following measuring equipment was used to investigate its qualities. It comprises an old valve potentiometer which has been superseded here for general use by a modern instrument. The circuit is essentially that due to Harrison (1930) somewhat modified; only one galvanometer is used; for standardization of the potentiometer the standard cell with a protective resistance is switched in place of the glass electrode. The potentiometer has four decades of studs giving a total of one volt by steps of 0·1 mv. The enclosed mirror galvanometer is deflected one division (2·5 mm.) for a change in the grid potential of 0·3 mv. Thus the instrument can produce and record changes of 0·1 mv. without interpolation on a coiled slide-wire or undue eyestrain in reading the meter. Since any convenient position on the galvanometer scale serves as zero, there is no need for frequent corrections to the meter for heater battery drift or for the "assymetry potential" of the electrode. All that is measured is the potentiometric difference between two solutions of which the pH of one is known. Such an arrangement is approved by Schwab (1958) in his comprehensive review. It reduces to a minimum the manipulations during a determination and avoids certain assumptions in the use of conventional pH meters.

The constant temperature bath consists of a clear, colourless glass jar about 11 cm. internal diameter and 17 cm. high. It stands in a galvanized iron case 20 cm. square and 19 cm. high which is packed with sawdust. The jar is supported with its top 3 cm. above the top of the case so that the electrode can be observed *in situ*. The jar is covered with a flat piece of "Acrylate sheet" 18 cm. wide by 15 cm. by 6 mm. thick. On it are mounted the electrode stand, the electrode vessel with its calomel half-cell, the heater, thermo-regulator and thermometer. The heater coil in the bottom of the jar is of "nichrome" wire 0·3 mm. thick having a resistance of 24 ohm. It is connected to a transformer giving 12 volts. The thermo-regulator is a small laboratory-made mercury-in-glass one which operates a thermionic relay. The current is alternately on and off for about 22 secs. each, giving a range of 0·015° C. The bath is filled to 1·5 cm. from the top with kerosene which is stirred with a rotary stirrer driven by vacuum from a water-pump. By thermo-couple measurements it was found that the liquid in the electrode vessel reaches a temperature 1° C. below that in the bath within 3-4 minutes after being put there and thereafter remains virtually constant. The bath is therefore kept at 39° C. In the bath are three small flasks, each holding 20 ml. whose necks protrude through and are clipped to the top of the bath in

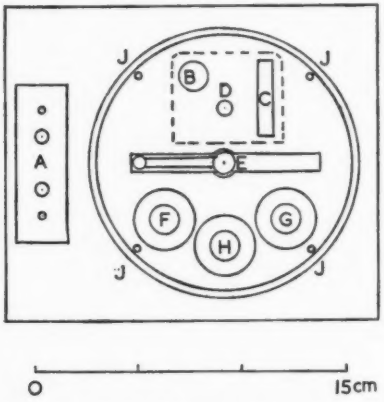

Fig. 1. Lay-out of the top of the apparatus.
A: Base of the electrode stand.
B: Opening for the heater leads in a glass tube.
C: Opening for the stirrer.
D: Opening for the thermometer.
E: Opening for the electrode vessel and for the thermo-regulator at the right-hand end.
F, G, H: Short-necked flasks for holding the buffer solutions.
J: Four positioning studs.

front of the electrode vessel. They hold the warm buffers for use in the determinations and are so shaped that the bent capillary pipettes can reach to their bottoms.

The back of the case is 38 cm. high and the shielded lead to the valve grid switch is anchored to it and passes through it, insulated from it. The lid is hinged to the top of the back and when it is lowered it completely covers the apparatus down to the top of the main case. The lid is raised and tilted back against a stop to expose the apparatus in order to fill the electrode vessel. The case, one terminal of the heater for the bath, the mercury in the thermo-regulator and the case of the valve potentiometer are "earthed" to the nearest water pipe, as are the valve-case and the negative heater-terminal of the valve.

The electrode vessel and calomel half-cell are shown in Fig. 2. The dimensions of the former were so chosen as to restrict the surface. Chemical tests showed how difficult it is to rid a large, deep, electrode vessel of one buffer before introducing another; this was amply confirmed in practical pH measurements. The inside of the whole unit was carefully siliconed. The wick consists of a loop of soft cotton string so chosen that about 0·1 ml. of

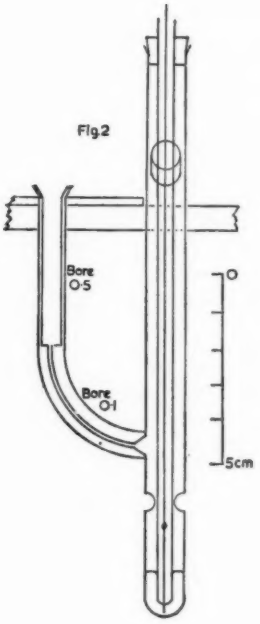

Fig. 2. Electrode vessel and calomel half-cell. The filling tube is at 45° to the vertical. Above the "Acrylate sheet" top is an apron of oil-resistant rubber about 2 mm. thick fitting snugly round the vessel.

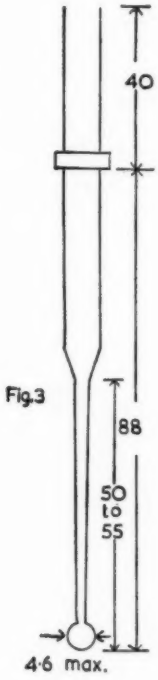

Fig. 3. Glass electrode; the maximum diameter of the bulb is 0·4 to 0·5 mm. less than the bore of the electrode vessel. Dimensions are in millimetres.

the 3·5 M KCl passes through during 24 hours when the level of the liquid in the tube above the half-cell is just below the opening in it. Tests showed that this simple device is stable enough during a determination at least for all ordinary biochemical purposes where small volumes of fluids are concerned. The wick is placed in position as follows: a doubled length of copper wire 0·17 mm. diameter is passed, loop foremost, from the electrode vessel through the capillary into the tube above the half-cell. It is drawn to the top of the tube and through its loop is passed the cotton about 16 cm. long. The ends of the cotton are knotted together forming a double strand about 8 cm. long. Through the cotton strand is passed another piece of cotton thread 20-30 cm. long, doubled. By pulling on the doubled copper wire the cotton strand is gently drawn into place in the capillary tube till it just projects into the electrode vessel. Holding the doubled long cotton thread and releasing one end of the copper wire, the latter is slowly drawn out of the cotton strand. The strand is then pulled cautiously back till its end is flush with the top of the capillary tube which forms the base of the electrode vessel. The long thread is then gently withdrawn from the cotton strand which is dropped loosely into the tube. For renewal the strand is readily removed with a small hook on the end of a steel wire. For this operation the electrical connection to the mercury pool is removed, but the pool itself with its calomel layer need not be further disturbed. The electrode vessel is welded to the capillary without spreading the latter at the join. The B.P. calomel and the mercury were ground together dry (Hills and Ives, 1951).

Glass electrodes have come to be regarded as long-lived, if slightly expensive, items of biochemical equipment. Various strenuous methods of cleaning them are prescribed, but Guthe (1959) has summarized their brief and unhappy lives when they are used in proteinous fluids. His experiences are sufficiently familiar to many precise workers. Wilkinson (1959) proposes a long capillary electrode of a special proprietary glass which might be bought in quantities and replaced as it ages. It seems somewhat fragile and must be cleaned daily with pepsin and HCl. For the preparation of glass electrodes that may be discarded when elderly without causing financial repercussions, the following scheme has proved to be practicable here. Some of the ideas were obtained from Charlton's (1956) paper, but the equipment used here is more generally accessible than his. Lengths of soda glass tubing 15 cm. by 6·6 mm. outside diameter are "thickened up" in the middle in a large bunsen or blowpipe and then drawn out to form thick-walled capillaries of 1 to 1·5 mm. outside diameter. In a small jewellers' oxy-coal gas torch clamped to a stand a bead of electrode glass (Corning 015 or its equivalent) is fused on a Pt wire. The warmed capillary is touched with the molten bead and withdrawn with its orifice sealed with a tiny bead of the special glass. This is heated in the torch and blown to a bulb. The ring gauge for determining the maximum diameter of the acceptable bulbs was 4·6 mm. The other dimensions of the electrode are shown in Fig. 3. After a little practice one finds a fair proportion of a batch with resistances in the suitable range of 200-500 megohms. Some, due to contamination of the electrode glass with soda glass, show too small a potentiometric difference between two known buffers and have to be discarded. Usable glass electrodes had about the same pH-voltage relationship as Wynn and Ludbrooke (1957) found for their larger ones. The electrodes are filled with 0·1 N HCl by immersion and evacuation 2-3 times. The rubber ring on the electrode stem holds the bulb 2 mm. above the liquid junction. Above the ring the stem is coated with a beeswax-resin cement. The Ag-AgCl electrode consists of a Ag wire sealed through a short piece of glass tube that slides into the stem of the glass electrode. The interior of the short tube is filled with the cement and the protruding Ag wire is chlorided electrolytically. The same cement holds the Ag-AgCl electrode in place. Part of this study was carried out with small 0·1 M KCl calomel half-cells in the glass electrode because of the fragility of the AgCl coat, but the results were the same. The electrode is loosely held in a hole in one end of a "Perspex" block on which

is mounted a terminal connected to the grid switch. The other end of the block is clamped to a small platform which can be raised or lowered on a pair of brass rods parallel to the axis of the electrode vessel. A spring clip holds the platform at the top of the rods. The copper wire connecting the electrode to the terminal is of 0·15 mm. diameter and has a few small coils in it to prevent it from straining the electrode system.

The buffers used include the phthalate and phosphate buffers described by Manov (1947) prepared from salts purified in this laboratory. Astrup's (1956) suggestion of a buffer nearer to the pH of normal blood was accepted; one containing 0·0125 M KH_2PO_4 and 0·0375 M Na_2HPO_4 was prepared for this purpose. In view of the statement by Wynn and Ludbrooke (1957) that such buffer solutions, in contrast to that containing 0·025 M KH_2PO_4 and 0·025 M Na_2HPO_4, are unstable and not to be relied upon, the following trial was made. 500 ml. of the above alkaline phosphate buffer were made with carefully dried salts. These were weighed into tared bottles in slight excess of their required amounts. They were then redried in the bottles and adjusted to the correct amounts from which the buffer solution was made. This was kept in a cold-room and small amounts were occasionally withdrawn. Six weeks later another lot of the same buffer was similarly prepared; when the two were compared with a large glass electrode the difference between them did not exceed 0·1 mv. It appears that the buffer is sufficiently stable when appropriately handled and is reproducible when prepared in a suitably equipped laboratory. Since an authoritative value for the pH of this buffer was not available here a sample was compared at 20° C. with the standard Manov phosphate buffer prepared here using a large electrode. They were also compared at 38° C. in the apparatus described. According to Astrup (1956) the pH at 38° C. of an alkaline phosphate buffer is rather uncertain. The value obtained here can serve as an internal reference until an authoritative figure determined with adequate equipment becomes available.

Little reliance can be placed on some makes of buffer salts as purchased. Among other incidents in this work an expensive and elaborately labelled lot of potassium hydrogen phthalate contained 4 p.c. of water and one of "anhydrous" di-sodium phosphate 8 p.c.

In operating this apparatus, the following conditions have been aimed at: cleanliness of the electrode and vessel (i.e. removal of a solution before putting in another), temperature control, maintenance of the liquid junction and avoidance of small air bubbles. These last are in all pH measurements a frequent cause of annoyance and betray themselves by a drift in the measurements at a rate of 1-2 mv. a minute. The following procedure was gradually found to be suitable. The electrode is raised and rinsed with about 1 ml. of the appropriate warm buffer, dripped on with a capillary pipette into a small plastic spoon. The electrode is then wiped with a fresh bit of filter paper about 20 by 15 mm. The electrode vessel is filled thrice with the same warm buffer and emptied; 0·2 ml. of the buffer is carefully put in the bottom of the vessel and observed to see that no air bubble is present; the electrode is gently lowered into place and again observed. The bottle of the buffer is re-stoppered and the lid lowered. A reading is at once taken and a stop-watch started. At each minute for the next six minutes a further reading is taken. It was found that thus the electrode and vessel were cleaned without unduly impairing the temperature. Thermo-couple measurements showed that even if the sample consisted of 0·2 ml. of ice-cold fluid the temperature was sufficiently recovered within 3-4 minutes. The reading after five minutes was accepted unless a drift of 0·3-0·5 mv. a minute was occurring when the determination was repeated. The usual drift is less than 0·1 mv. per minute.

The capillary pipettes are bent at 30° at 5 cm. from the tip which must be flame polished. Just above the bend is a small bulb so that the total volume to the top of the bulb is about 0·2 ml. A separate one is kept for each buffer solution. A similar pipette or a syringe with a bent, blunt needle, according to circumstances, is used for introducing the blood.

RESULTS.

The sort of results to be obtained from the apparatus can be gathered from Tables 1 and 2. The temperature was 38° C. The buffers were put in the electrode vessel in the order from left to right. The readings are in mv. and were taken at one minute intervals after putting the electrode in the fluid.

TABLE 1.

Buffer	Manov phosphate	Alkaline phosphate	Manov phthalate	Manov phosphate	Alkaline phosphate	Manov phthalate
	287·3	314·4	114·7	287·9	314·6	115·5
	287·4	314·9	115·2	288·1	315·1	115·8
	287·5	314·9	115·3	288·2	315·2	115·8
	287·6	314·6	115·2	288·1	315·1	115·9
	287·7	314·6	115·3	288·2	315·2	115·8
	287·7	314·6	115·3	288·2	315·3	115·8
	287·8	314·7	115·4	288·2	315·3	115·8

TABLE 2.

Fluid	Manov phosphate	Alkaline phosphate	Blood	Blood	Alkaline phosphate	Alkaline phosphate	Manov phosphate
	284·3	311·4	319·3	319·5	311·9	312·2	284·6
	284·6	311·8	319·1	319·1	312·4	312·6	284·8
	284·7	311·9	319·1	319·0	312·6	312·6	284·8
	284·8	312·0	319·1	319·0	312·4	312·6	284·9
	284·9	312·1	319·1	318·9	312·5	312·6	284·9
	285·0	312·2	319·2	318·9	312·6	312·6	284·9
		312·3	319·2	318·9	312·6		

Different electrodes were used for the two Tables. Each column refers to a fresh lot of fluid in the electrode vessel. The readings were taken at one minute intervals and the ones after five minutes used for the calculations. The pH of the alkaline phosphate was 7·28 from Table 1 and 7·29 from Table 2; the two samples of the same blood were 7·40 and 7·39 respectively. Such differences indicate the limit of accuracy of the instrument. The determination at 20° C. with a large electrode of the pH of the alkaline phosphate indicated a pH of 7·29 for it at 38° C.

The use of an open electrode vessel (Wynn, 1957) appears to be justified by the stability of the figures for blood. The small electrode drift was noticed by the same author as a general property of such small electrodes.

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STUDIES OF ARTHROPOD-BORNE VIRUS INFECTIONS IN QUEENSLAND

II. SEROLOGICAL INVESTIGATIONS OF ANTIBODIES TO DENGUE AND MURRAY VALLEY ENCEPHALITIS IN EASTERN QUEENSLAND

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(*Accepted for publication 5th July, 1960.*)

SUMMARY.

Serological studies of 62 patients with clinical dengue in the 1953-55 epidemic in North Queensland demonstrated the diagnostic value of the haemagglutination-inhibition test against Murray Valley encephalitis antigen. Two distinct patterns of response to complement-fixation, haemagglutination-inhibition, and neutralization tests were demonstrated, and are considered to represent first and second infections with dengue.

The age distribution of antibodies suggested that about 40 p.c. of the non-immune population of Brisbane were infected in the 1942-44 epidemic of dengue. No evidence was found of more recent dengue infection in Brisbane, but one patient from Cooktown had evidence of dengue infection between 1947 and 1953, when no epidemic was recognized.

Neutralization tests demonstrated that the human immunity pattern to group B arthropod-borne viruses at Brisbane, Townsville, Innisfail, the Atherton Tableland and Lockhart River Mission was due to infection with dengue, and not with MVE. However, evidence of MVE infection at Townsville since 1956 was found in fowl sera. Sera from Coen and Wrotham Park showed evidence of infection with MVE, and not with dengue.

Evidence is presented that the 1925-26 and 1953-55 epidemics of dengue in Queensland were due to dengue type 1, and the 1942-44 epidemic predominantly to dengue type 2, but with some cases due to dengue 1. It was necessary to use suckling mice to determine neutralization indices in order to obtain a type-specific response.

INTRODUCTION.

A previous survey (Doherty, Carley and Lee, 1959) demonstrated that a high proportion of aboriginal children on missions bordering the Gulf of Carpentaria had antibodies to Murray Valley encephalitis (MVE). The small number neutralizing dengue 1 or dengue 2 was thought to indicate cross-reaction from MVE rather than infection with dengue. In the present paper, serological studies are reported on patients who had clinical attacks of dengue fever in the epidemics of 1926, 1942 or 1954, and on survey samples from several population groups on or near the east coast of Queensland.

MATERIALS AND METHODS.

The strains of MVE, dengue 1, and dengue 2 used, and the methods of performing haemagglutination-inhibition (HI) and neutralization tests, were described previously (Doherty *et al.*, 1959). The haemagglutinin content of acetone-ether or sucrose-acetone extracts (Clarke and Casals, 1958) of suckling mouse brain infected with dengue 1 or dengue 2 was consistently low, in contrast to the high titre preparations obtained from MVE. A number of Queensland sera were tested for HI antibodies against all three antigens; no evidence was found that the antigens differed significantly in their ability to detect group B antibody, and MVE antigen was therefore used routinely.

Neutralization indices were determined by testing undiluted sera, heated at 56° C. for 30 minutes, against serial tenfold dilutions of virus in 10 p.c. rabbit serum-saline. Virus-serum mixtures were incubated at 37° C. for one hour, and inoculated intracerebrally into three-week-old mice (dengue 1 and dengue 2), or intraperitoneally into five- to eight-day-old mice (MVE), as in screen tests against a single dose of virus. Neutralization indices were also determined against dengue 1 and dengue 2 on a number of sera, by inoculating virus-serum mixtures intracerebrally into two- to four-day-old mice. Titres were calculated by the method of Reed and Muench (1938). The neutralization index was the difference between logarithms of virus titres in the presence of the serum on test, and in the presence of normal inactivated rabbit serum.

Neutralization titres were determined by testing serial $10^{0.5}$-fold dilutions of inactivated serum in normal rabbit serum against a dose of virus calculated to give 100 LD_{50} in the final inoculum.

RESULTS.

Sera from patients with dengue in 1954-55.

Sera were available from 70 of 75 patients with illnesses diagnosed clinically as dengue at the Innisfail Field Station of the Institute during an epidemic in 1954-55 (Doherty, 1957). These sera were tested, as permitted by the amounts available, for neutralizing antibodies to dengue 1, dengue 2, and MVE, and for HI antibodies to MVE. The sera had previously been tested for complement-fixing (CF) antibodies to MVE by Ludford and Cook (1957), and their titres are quoted here. The diagnosis was confirmed in 52 patients, four were negative to all tests, and 14 showed evidence of previous infection, not related to the illness under investigation. Paired sera from 206 other patients with febrile illnesses studied at the Field Station between 1952 and 1955 were tested for HI antibodies to MVE. Ten had rising titres, shown by neutralization tests to be due to dengue infection.

Typical results obtained from the 62 patients finally accepted as having dengue are shown in Table 1. The contribution of the tests towards establishing a diagnosis is summarized in Table 2 for 32 patients, paired sera from whom were tested by the four techniques. The HI test was more useful than the neutralization test against single doses of dengue 1 or dengue 2. Dengue 1 neutralization test was more efficient than dengue 2. Neutralization titres determined on sets of paired sera (Table 5) showed rising titres in several pairs in which both sera were positive to initial screen test.

TABLE 1.

Results of serological tests on paired sera from patients with dengue fever, 1954-55.

Patient	Age	Days after onset	MVE HI	MVE CF	Neutralization test		
					dengue 1	dengue 2	MVE
E.W.	5	4	neg.*	5	neg.	neg.	
		42	40	5	pos.	neg.	
S.F.	5	3	neg.	neg.*	neg.		neg.
		17	160	neg.	neg.	neg.	neg.
N.K.	15	2	neg.	neg.	neg.	neg.	neg.
		44	160	neg.	pos.	pos.	neg.
B.R.	20	5	neg.	neg.	neg.	neg.	neg.
		61	160	neg.	pos.	pos.	neg.
A.B.	13	2	neg.	neg.	neg.	pos.	neg.
		25	640+	160	pos.	pos.	pos.
B.G.	15	2	neg.	neg.	neg.	pos.	neg.
		20	640+	320+	pos.	pos.	pos.
D.S.	62	5	160	5	pos.	pos.	pos.
		28	640+	160	pos.	pos.	pos.
R.M.	22	2	neg.	neg.	pos.	pos.	neg.
		22	640+	320+	pos.	pos.	pos.

* Titres expressed as reciprocal. Negative = <10(HI) or <5(CF).
\+ Highest dilution tested.

TABLE 2.

Comparison of diagnostic tests on paired sera from 32 patients with dengue, 1954-55.

Test	Virus	Number of patients with:		
		Rising titres	High titres in both sera	Negative in both sera
HI	MVE	25	5	2
CF	MVE	13	2	17
Neutralization (screen tests)	dengue 1	21	10	1
	dengue 2	11	11	10

The patients may be divided into two groups on the basis of the CF test against MVE (Table 3). Thirty-one patients (e.g. the last four patients in Table 1) developed CF antibodies to titres of 20 or greater in convalescence. They also showed a rapid development of MVE HI antibodies to high titre, and most had neutralizing antibodies to dengue 2 in the first serum tested. Twenty-six patients who developed no MVE CF antibodies, or developed them only in titres of 5 or 10, showed a slower response in lower titre by HI test. No acute serum from this group neutralized dengue 2, and only one neutralized dengue 1.

None of five patients aged under 10 years developed CF antibodies to MVE,

but 11 of 21 aged 11-20 years and nine of 13 aged 21-30 years did. Neutralization indices (Table 4) and titres (Table 5) were determined on a number of convalescent sera. Neutralization titres were done in addition to the more

TABLE 3.

Differences in serological response between MVE CF-positive and CF-negative patients.

	MVE CF-positive (31 patients)	MVE CF-negative (26 patients)
Dengue 1 neutralizing antibodies in acute serum*	9/19	1/22
Dengue 2 neutralizing antibodies in acute serum*	13/16	0/21
MVE neutralizing antibodies in acute serum+	5/13	0/13
MVE neutralizing antibodies in convalescent serum*	15/22	2/21
MVE HI antibodies by 5th day after onset*	19/24	3/20

* $P<0{\cdot}01$ for difference between groups.
\+ $P<0{\cdot}05$ for difference between groups.

TABLE 4.

Neutralization indices of convalescent sera from patients with dengue in 1926, 1942, or 1954 epidemics.

Epidemic	Patient	Time after onset	dengue 1	dengue 2
1926	A.L.	32 years	3·9*	1·9
	M.L.	32 years	4·0 (3·7)	4·0 (1·3)
	J.W.	32 years	2·4 (2·1)	3·5 (<1·8)
	G.C.	32 years	3·0 (3·4)	3·0 (<1·3)
	E.D.	32 years	3·9 (2·9)	2·9 (<1·3)
1942	I.C.	15 years	1·6 (1·3)	2·6 (2·3)
	D.H.	16 years	2·9	<1·9
	D.S.	16 years	<1·7 (1·6)	3·0 (2·2)
	P.L.	16 years	2·8 (2·0)	3·0 (<1·8)
	E.S.	17 years	(1·6)	(2·6)
	L.D.	17 years	(2·6)	(2·7)
	J.L.	17 years	(<1·0)	(2·7)
1954	B.G.†	20 days	4·3 (3·7)	>4·5 (2·3)
	R.M.†	22 days	4·0 (3·7)	(2·3)
	M.R.†	121 days	3·2 (2·4)	4·6 (2·2)
	M.C.†	1246 days	2·8	4·4
	J.C.†	17 days	(3·4)	(2·2)
		1296 days	3·0 (1·7)	3·4 (≤0·5)
	P.W.†	19 days	3·8 (2·9)	2·6 (3·2)
	L.D.†	41 days	2·9 (3·3)	3·9 (2·2)
	J.B.	1401 days	2·5 (2·5)	2·2 (<1·4)
	Y.S.	48 days	2·4 (2·1)	2·8 (<1·4)
	L.C.	105 days	2·4 (2·4)	1·8 (<1·4)
	J.S.	25 days	2·1 (2·1)	1·8 (<1·4)

* Log_{10} neutralization index. Figures in brackets give index determined on same serum in suckling mice. Sera were used undiluted in adult mice, but diluted 1/2 in suckling mice.
† MVE CF-positive cases.

common neutralization indices, because the technique required less serum, and because several authorities (Lennette, 1956; Tyrrell and Horsfall, 1953) have considered the neutralization titre to give a better quantitative estimate of antibody than the neutralization index. The antibody levels to dengue 1 and dengue 2 were higher in patients who showed an MVE CF response ($P < 0{\cdot}05$ for dengue 1 neutralization indices in adult mice).

Neutralizing antibodies to MVE developed in most of the CF-positive patients. Three, who had no MVE antibodies in their acute sera, had neutralization titres of the same order to MVE, dengue 1 and dengue 2 in their convalescent sera (Table 5). A limited amount of information was obtained about the duration of the MVE neutralizing antibodies; while three patients who developed MVE antibodies early in their illnesses had none demonstrable 41 to 78 days after onset, three of five others still had neutralizing antibodies to MVE four years after onset.

For reasons discussed below, the CF positive and negative patients are considered to represent second and first infections with dengue respectively. A search was made for sera collected in previous illnesses from the CF positive patients. Two were found, collected one and three years before the illnesses under investigation. Both neutralized a screening dose of dengue 2.

Neutralization tests against screening doses of the dengue viruses indicated that the epidemic virus was more closely related to dengue 1 than to dengue 2

TABLE 5.

Neutralization titres of paired or single convalescent sera from patients with dengue, 1954-55.

Patient	Days after onset	dengue 1		dengue 2		MVE	
		Virus* dose	Titre	Virus* dose	Titre	Virus* dose	Titre
A.B.†	2	63	6	200	10	40	<1
	25	63	>100	200	>100	56	66
L.D.†	5	63	>100	200	>100	100	>1
	41	63	>100	200	>100	100	<1
C.M.†	3	63	>100	200	>100	159	>1
	49	63	40	200	>100	159	<1
J.C.†	3	63	3	200	<1	40	<1
	17	63	>100	200	100	56	72
D.S.†	5	63	<3	200	5	159	>1
	28	63	6	200	12	159	>1
B.G.†	2			126	>1	500	<1
	20	40	100	17	>300	40	160
R.M.†	22	40	200	50	>300	160	12
E.C.	3	63	<3	200	<1	40	<1
	50	63	<3	200	<3	40	<1
Y.S.	48	30	<3	50	4	100	<1
L.C.	105	40	5	17	6	40	<1
B.R.	61	63	<3	200	<3	159	<1

* LD_{50} of virus against which serum dilutions were tested.
† MVE CF-positive.

(Table 2). Neutralization indices were determined on a group of convalescent sera that had neutralized both viruses in screen tests (Table 4). The sera from the MVE CF positive group had high indices to both viruses, higher in most to dengue 2. The sera from MVE CF negative patients had, in general, a higher response to dengue 1, although in one the dengue 2 index was higher. Convincing differences in indices to dengue 1 and dengue 2 were not demonstrated in either group ($P > 0{\cdot}10$). Similarly, neutralization titres did not distinguish between the two viruses (Table 5). However, when neutralization indices were determined by inoculation of suckling mice (Table 4), a much clearer result was obtained; indices were higher to dengue 1 in both groups ($P < 0{\cdot}02$), and the MVE CF negative group neutralized only dengue 1.

Survey of population groups in Queensland.

Paired sera from patients with a variety of febrile illnesses, collected between 1952 and 1955 at Innisfail, and between 1957 and 1959 at Brisbane, were tested for HI antibodies to MVE. No antibody rises were detected, except in the cases of dengue considered above. Positive titres were taken to indicate prior infection, and are analysed according to age and locality in Table 6. Only one of each pair of sera is represented in the Table. Single sera, collected as survey samples from various groups, are also included. Most HI positive, and a sample of HI negative, sera were tested for neutralizing antibodies to dengue 1, dengue 2, and MVE (Table 7).

TABLE 6.

Age distribution of MVE HI antibodies.

Locality	Years collected	Age group					
		0-10	11-20	21-30	31-40	40	Total
Brisbane	1957-59	0/36*	5/20	13/33	8/13	27/41	53/143
		(0)†	(25)	(39)	(62)	(66)	(37)
Innisfail	1952 to March, 1954	6/39	13/40	15/36	11/18	15/18	60/151
		(15)	(32)	(42)	(61)	(83)	(40)
Innisfail	March, 1954 to September, 1955	6/25	5/23	10/28	11/18	19/22	51/116
		(24)	(22)	(36)	(61)	(86)	(44)
Lockhart River Mission	1956	0/2	13/24	5/11	5/5	8/10	31/52
			(54)	(45)			(60)
Atherton Tableland	1959	0/1	7/21	7/14	8/10	12/17	34/63
			(33)	(50)		(71)	(54)
Coen and Wrotham Park	1959		1/2	3/5	3/3	8/8	15/18
							(83)
Townsville	1957-59	1/1		2/3	4/4	39/39	46/47
						(100)	(98)

* Number positive at dilution 1:10 or greater/number tested.
† Percentage positive.

TABLE 7.

Correlation of HI and neutralization test results.

Locality	HI-positive			HI-negative		
	dengue 1	dengue 2	MVE	dengue 1	dengue 2	MVE
Brisbane	35/47	30/38	16/43	6/35	2/36	1/30
Innisfail (1952 to 1954)	35/47	32/36	9/46	1/12	0/9	0/7
Innisfail (1954 to 1955)	36/51	26/35	10/47	1/39	3/33	0/10
Lockhart R. Mission	19/30	5/29	5/29	1/17	0/17	0/16
Atherton Tableland	26/35	26/30	14/34	5/21	2/21	0/20
Coen and Wrotham Park	3/15	3/15	13/15	0/3	0/3	3/3
Townsville	39/41	31/36	10/44			

Brisbane. No evidence of dengue or MVE infection was found in children born after the 1942-44 epidemic of dengue. The sera of the 36 children aged 0-10 in Table 6 were supplemented by 63 sera from children aged 12 or less, collected in the Brisbane-Ipswich area in 1958-59 by the Laboratory of Microbiology and Pathology, Department of Health and Home Affairs, Brisbane. All were negative, as were 50 sera collected from children at Toowoomba in 1959. Sera were also obtained from the Laboratory of Microbiology and Pathology from Brisbane patients aged 13-30; 18 of 50 had HI antibodies, which is in agreement with the proportion in Table 6.

The correlation between neutralization and HI tests is of the same order as found in the previous survey. A much higher proportion of sera neutralized dengue than MVE. Detailed inspection shows that all sera that neutralized MVE also neutralized one or both dengue viruses, with only two exceptions, each of which showed partial protection against dengue 2.

Innisfail. The sera from the Innisfail Field Station have been considered in two groups, collected before or after March, 1954, when the most recent epidemic of dengue appeared in the area. Patients with proven dengue all came from the later period, and are excluded from this series. It is evident that the age distribution of HI antibodies shows little difference between the two groups, suggesting that there was not any large amount of unrecognized infection in the community.

Sera were tested from 21 children born after 1945, and bled before 1954, and therefore not exposed to a recognized epidemic. One child, born in 1947, had MVE HI antibodies and neutralizing antibodies to dengue 1 in both sera collected during an illness diagnosed serologically as Q fever in 1953. He had lived near Cooktown all his life.

Other areas. All Innisfail sera that neutralized MVE also neutralized one or both dengue viruses. This is in contrast to the situation previously demonstrated near the Gulf of Carpentaria (Doherty *et al.*, 1959). Sera were tested

from several other areas in an attempt to determine the geographical limits of the east coast and Carpentaria patterns of immunity.

Survey samples collected at Lockhart River Mission on the east coast of Cape York Peninsula, and at Mareeba, Atherton and Herberton on the Atherton Tableland, all had a high proportion positive to the MVE HI test. Neutralization tests showed that dengue immunity was dominant. All sera that neutralized MVE also neutralized dengue with the single exception of a 22-year-old nurse from Atherton, who had lived at Atherton, Innisfail or Gordonvale all her life, and whose serum neutralized MVE but not dengue 1 or dengue 2.

In contrast, sera collected at Coen and at Wrotham Park (near Chillagoe) had an immunity pattern predominantly to MVE. Only one patient neutralized both dengue viruses but not MVE. She had lived at Cooktown for some years, and could have been infected with dengue there.

Two epidemics of X disease have been described at Townsville (Breinl, 1918; Baldwin and Heydon, 1925). These are considered to have been due to MVE (Anderson, 1954). A group of patients who had been resident in Townsville in the period 1917-25 was bled in July, 1959. These sera, and other Townsville sera submitted from patients with various illnesses, had a very high rate of immunity to dengue. The number neutralizing MVE was much smaller, but the number of sera without dengue antibodies was so small that no conclusions could be drawn. Five of 21 fowl sera collected at Townsville in July, 1959, had both HI and neutralizing antibodies to MVE; one of the five neutralized dengue 1, and none neutralized dengue 2. Three of the HI-positive fowls were eight years old, one between three and five, and one three years.

A group of sera, previously found to neutralize MVE in baby mice inoculated intraperitoneally, was retested by a similar technique using weaned mice inoculated intracerebrally. Eleven of 12 sera from missions where MVE immunity was dominant (Doherty *et al.*, 1959) neutralized MVE by this test, whereas only eight were positive of 33 from east coast areas where dengue immunity predominated. Five were positive of 13 convalescent sera from patients with dengue in 1954. A comparison of neutralization indices by the two tests showed that, for sera in each group, the test in baby mice gave indices tenfold higher.

Antigenic typing of successive dengue epidemics.

Data relevant to the antigenic type of virus responsible for the 1954-55 epidemic have been detailed above.

It seems reasonable to assume that most, if not all, of the dengue immunity demonstrated in sera from Brisbane and Innisfail was due to infection in the major epidemics of 1925-26, 1942-44, and, in Innisfail only, 1953-55. This allows a number of sera with antibodies to be allotted to one or other of the two most recent epidemics, according to the age of the donor and the date on which serum was collected. Thus, Brisbane patients aged 13 to 30 in 1957-59 were exposed

TABLE 8.

Pattern of neutralization response in screen tests on survey sera from patients exposed to only one epidemic of dengue.

Epidemic	Virus neutralized		
	dengue 1 only	dengue 2 only	both dengue 1 and dengue 2
1942-44	5	15	14
1953-55	11	1	15

only to the 1942-44 epidemic. Table 8 summarizes the results thus obtained from screen tests against dengue 1 and 2. Fifteen of 34 neutralized dengue 2 only, five dengue 1 only. For comparison, 1954-55 patients are included whose acute sera had no antibodies to either virus, or whose age made it impossible for them to have been infected in 1942-44. A few survey sera from North Queensland children, bled in 1957-59 and not discussed elsewhere in the paper, have been included in this group.

Neutralization indices against dengue 1 and dengue 2 were determined on sera from patients with definite histories of dengue in the 1926 or 1942 epidemics. Tests of the sera still available were later repeated, using suckling mice, and a more specific response was obtained (Table 4). The 1926 patients showed antibodies predominantly to dengue 1 ($P < 0{\cdot}05$). The 1942 group included patients with responses to both viruses, to dengue 2 only, and to dengue 1 only.

DISCUSSION.

Ludford and Cook (1957) demonstrated MVE CF antibodies in a proportion of convalescent sera from Innisfail patients with dengue in 1954-55. Rowan (1959) studied a group of patients at Townsville in the same epidemic. He confirmed the presence of CF antibodies to MVE, and also demonstrated CF antibodies to dengue 1, dengue 2, Japanese B encephalitis and West Nile virus. He determined neutralization indices to all five viruses on a group of convalescent sera, and found high indices to both dengue viruses, higher in most to dengue 1, but little evidence of neutralization of MVE, Japanese B encephalitis or West Nile virus. He concluded that the epidemic was due to dengue 1, but that previous infection with dengue 2 may have modified the serological response. He was unable to test acute sera to confirm this, but recorded that several patients had histories of dengue in both 1942 and 1954 (Rowan, 1956).

The present study extends these findings in several ways. The MVE HI test was demonstrated to provide a useful diagnostic test, as was expected from the group-specific nature of the HI response (Casals and Brown, 1954). The division of patients into groups on the basis of the MVE CF test results revealed

two distinct patterns of serological response. The hypothesis that these represent first and second infections with dengue is supported by the presence of dengue neutralizing antibodies in the acute sera of the CF positive group, but only rarely in the CF negative group, and, in two cases, by the presence of neutralizing antibodies to dengue 2 in sera collected before the illness under investigation. It is noteworthy that the five children with dengue in 1954, all too young to have been exposed to infection in 1942-44, showed the serological response attributed to primary infections.

Hotta (1952) found that two volunteers with previous histories of dengue developed antibodies more rapidly than two with no previous history of dengue when challenged with a strain of dengue 1. Differences in serological response following first and second infections with group B arthropod-borne viruses have been described for man and experimental animals by a number of workers (reviewed by Casals and Reeves, 1959). Theiler and Casals (1958) studied a group of patients with yellow fever, diagnosed by virus isolation, and demonstrated two patterns of response, associated with presence or absence of evidence of previous infection with Ilhéus or dengue viruses. The characteristic response to second infection described by these workers, rapid production of homologous antibodies and the production of heterologous CF and neutralizing antibodies, is identical with those of the MVE CF positive patients in the present series.

Most MVE CF positive patients in the present series also developed neutralizing antibodies to MVE, while Ludford and Cook (1957) and Rowan (1959) found little evidence of neutralization in convalescent sera. This discrepancy may be due to the techniques employed. In the present series of tests, virus-serum mixtures were incubated at 37° C. for an hour before inoculation intraperitoneally into suckling mice, while the other workers either did not incubate the virus-serum mixtures (Ludford and Cook) or inoculated weaned mice intracerebrally (Rowan). The importance of the route of inoculation and age of mice is confirmed by experiments reported above.

Dengue is a common diagnosis for short febrile illnesses between major epidemics in Queensland. Lumley and Taylor (1943) described it as occurring "frequently since 1926". However, the occurrence of large epidemics seems incompatible with regular endemic incidence (Derrick and Bicks, 1958). The present study gives no evidence of dengue infection in Brisbane after the last recognized epidemic in 1942-44. The HI and neutralization test results show that about 40 p.c. of the age groups of the population not exposed to the 1926 epidemic were infected in the 1942 epidemic. The evidence of dengue infection at Cooktown between 1947 and 1953, when no recognized epidemic occurred, is based on a single patient, but may indicate the persistence or occasional introduction of dengue or a related virus between epidemics in that area, and warrants further investigation.

The data analysed in Tables 2, 4 and 8 suggest that the 1926 and 1954 epidemics were due to dengue 1, but that the 1942 epidemic, while predominantly due to dengue 2, also included cases infected with dengue 1. This seems not unlikely, as during the war years there was heavy military traffic between Queensland and New Guinea, where dengue virus strains of both types were isolated (Sabin, 1952). These typings, based on serological data without virus isolation, must be accepted with some caution, especially for the 1926 epidemic. Retrospective typings of dengue epidemics have been reported by Sabin (1952) for epidemics in Japan in 1944-45 and Hawaii in 1943-44, by Kokernot *et al.* (1956) for the Durban epidemic of 1926, by Rosen (1958a, 1958b) for epidemics in the Society Islands in 1944 and Panama in 1941-42, and by Theiler *et al.* (1960) for the epidemic in Greece in 1927-28.

The clear distinction of antibodies to dengue 1 and dengue 2 when neutralization indices were determined by inoculation of baby mice, in contrast to the frequent cross-reactions when adult mice were used, was an unexpected finding. McLean and Magrath (1959), using baby mice, found no cross-reaction between dengue 1 and dengue 2 in screen tests on a group of sera from the Northern Territory. The difference between the two hosts warrants further study, and has obvious importance in future surveys.

The occurrence of MVE neutralizing antibodies in sera from Brisbane, Innisfail, Lockhart River and the Atherton Tableland only in the presence of antibodies to dengue, with very few exceptions, suggests that the immunity pattern demonstrated is due to dengue infection, and that the MVE antibodies are due to cross-reaction. The development of MVE antibodies demonstrated after second attacks of dengue is obviously relevant, and it is noteworthy that 28 of 35 patients from Brisbane and Innisfail with MVE antibodies were old enough to have been exposed to two epidemics of dengue. The greater frequency of cross-reaction demonstrated in neutralization tests by a peripheral route of inoculation (Parks and Price, 1958), as used in MVE tests in the present survey, may have been of importance also. Theiler (quoted by Casals, 1957) found that sera from children immune to dengue would protect mice against yellow fever when inoculated by the intraperitoneal route, but not by the intracerebral route.

It appears, therefore, that human immunities to dengue and MVE in Queensland have separate geographical distributions, MVE immunity being found around the Gulf of Carpentaria extending east to Coen and Wrotham Park, and dengue immunity along the east coast and on the Atherton Tableland. MVE is believed to be introduced and spread by migratory birds, with transmission by *Culex* mosquitoes (Anderson, 1954). Dengue is believed to be introduced by man into the ports, especially Townsville, and spread by the movement of infected cases, with transmission by *Aedes aegypti*. These differences would account for the general pattern of geographical distribution demonstrated, but would not explain the absence of geographical overlap to any significant degree.

MVE virus has been active on the east coast, as indicated by the results on fowl sera in the present study, and on horse sera reported by Ludford and Cook (1957). The failure to demonstrate MVE infection in man, in contrast to the high infection rate further west, may suggest that only small amounts of virus have been disseminated on the east coast. Beech *et al.* (1958) found a much higher rate of MVE immunity on an aboriginal mission in South Australia than in a neighbouring white community, and suggested that social factors might influence exposure to infected mosquitoes. The effect that immunity to one group B virus may have on the epidemiological behaviour and geographical distribution of other group B viruses in the same area has been discussed by a number of workers, most recently by Smith (1958) and Macnamara *et al.* (1959). The degree to which these factors have influenced the geographical distribution of MVE and dengue immunity in Queensland cannot be determined on the evidence available.

Acknowledgments. The authors are indebted to Dr. M. J. Mackerras and Miss M. L. Emanuel for the collections of sera from Lockhart River and the Atherton Tableland, to Dr. K. Pettit and the Royal Flying Doctor Service for making possible the collection of sera at Coen and Wrotham Park, to Dr. N. Scott-Young and Dr. R. Rimington for their assistance with the collection of sera at Townsville, to Mr. J. J. McLachlan, of the Department of Agriculture and Stock, Townsville, for his help in collecting fowl serum, and to Dr. E. H. Derrick for his assistance in locating people who had dengue in 1926.

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THE OCCURRENCE OF β-AMINO-ISOBUTYRIC ACID IN THE URINE OF PATIENTS WITH THALASSEMIA MAJOR

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(*Accepted for publication 7th July, 1960.*)

SUMMARY.

Three cases of thalassemia major in whom splenectomy had not been performed were found to be excreting large amounts of β-amino-isobutyric acid (BAIB).

Two cases of thalassemia major in whom splenectomy had been performed were found to be excreting much smaller amounts of this amino-acid.

The possible significance of BAIB in thalassemia major is briefly considered.

INTRODUCTION.

Dent (1948), by the use of chromatographic techniques, found in the urine of normal people an unidentified amino-acid which was subsequently shown to be β-amino-isobutyric acid or BAIB (Crumpler *et al.*, 1951; Fink, 1951a), an amino-acid not found in proteins. This paper describes a previously unreported association between thalassemia major and the excretion of BAIB.

Materials and Methods.

Specimens of urine and serum from three families of Mediterranean extraction were studied; each family had two children suffering from thalassemia major and of these six children, five were available for study. Two children had had splenectomy performed one and two years respectively before this investigation. Diagnosis was made from the clinical history and the results of clinical, radiological and haematological examinations. Foetal haemoglobin was estimated where possible in the children, and haemoglobin A_2 in the parents. The amino-acid pattern of the urine and serum of the affected children was examined repeatedly over a period of 18 months. Urine samples from the six parents and a total of four siblings of these children were studied also.

The technique of paper chromatography used for examination of urine and serum, and the units expressing amino-acid concentration are as described previously (Hillcoat, in press). The units are arbitrarily derived by comparing the intensity of colour of the amino-acid spots with the intensity of colour of a glycine spot of standard concentration.

To isolate the unidentified amino-acid that appeared in paper chromatograms of the urine a procedure similar to that of Fink (1951a) was adopted as follows:

The urine was initially purified by passing it through a column of Zeokarb-225 resin in the acid form, so that the cations, including the amino-acids, were absorbed from the urine on the resin. The column was then washed with distilled water and the cations eluted with ammonia.

The eluate was concentrated to a volume equal to that of the original urine sample.

A two dimensional chromatogram was prepared as previously described, using Whatman No. 1 paper and 80 p.c. ethanol as the first solvent and water saturated phenol for the second solvent. When developed with ninhydrin this chromatogram localised the position of the unidentified spot.

A sufficiently large volume of eluate to saturate the paper was streaked across the full width of a sheet of Whatman No. 3 paper and 80 p.c. ethanol used as a solvent with a descending technique. The paper was dried, thin vertical strips cut from each side were developed with ninhydrin, and a horizontal strip corresponding to the position of the unidentified amino-acid cut out. The material contained in this strip was eluted with distilled water and concentrated to a small volume.

This concentrate was streaked across the full width of a sheet of Whatman No. 3 paper and water saturated phenol used as a solvent with a descending technique. Vertical strips were cut as above and a horizontal strip eluted. This eluate was concentrated to a small volume.

The above isolation procedures were repeated many times and the resultant fractions were pooled. Evaporation under vacuum yielded crystalline material with some brown phenolic contaminant. The crystals obtained were morphologically similar to those of authentic BAIB. It was not possible to free the material from the contaminant so that identification was carried out by chromatographic techniques alone.

Identification of the isolated material.

Two dimensional chromatography of a solution of the material with 80 p.c. (v/v) ethanol-water, and water saturated phenol as solvents showed only one ninhydrin reacting substance to be present.

When solutions of authentic BAIB and of the isolated material were applied to the same spot and the above chromatographic procedure repeated, one ninhydrin reacting spot resulted.

Chromatography with three different one-dimensional systems, acidic (butanol-acetic acid-water), basic (butanol-pyridine-water), and neutral (ethanol-water) gave identical R_f values for the isolated material and authentic BAIB.

The colour reaction of the isolated material with ninhydrin after treatment with cupric carbonate (Crumpler and Dent, 1949) was identical with that of the sample of authentic BAIB and different from that of α-amino-n-butyric acid. α-amino-n-butyric acid gave no colour with ninhydrin after copper treatment.

The unidentified substance was stable to acid hydrolysis.

From the above data, the substance was considered to be BAIB.

RESULTS.

Table 1 illustrates the results obtained from urinary chromatograms of the patients studied. A comparison with a urinary chromatogram from a normal child shows: (1) the greatly increased excretion of BAIB in three of the five affected children; (2) the total amino-acid excretion is less than the upper limit of normal of 30 units previously described (Hillcoat, in press); (3) the pattern of amino-acids, apart from BAIB, is normal.

These findings were constant over a period of 18 months and were not influenced by infection, transfusions or the degree of anaemia and were apparently uninfluenced by diet. In the two children who had both undergone splenectomy BAIB was present in small amounts or was completely lacking.

With the technique used, no BAIB could be detected in the serum of any of the patients with thalassemia major, their parents or their siblings.

No urine samples from any of the six parents or from the four siblings of the affected children contained BAIB.

TABLE 1.

Urinary amino-acid patterns in patients with thalassemia major.

	Normal	M.T.	P.T.	R.M.	J.C.*	B.C.*
Glycine	3	3	4	3	4	4
Serine	2	2	3	2	3	3
Glutamine	3	3	4	3	4	3
Alanine	2	1	2	2	2	2
Threonine	1	0	0	1	1	0
Methyl histidine	0	0	1	0	1	1
Histidine	2	2	2	1	2	2
Valine	0	0	0	1	1	1
Tyrosine	0	0	0	0	0	0
Tryptophane	0	0	0	0	0	0
Taurine	0	2	0	0	0	0
Leucine & isoleucine	0	1	1	1	1	1
Aspartic acid	1	1	1	1	1	2
Glutamic acid	1	2	2	1	1	1
Cysteic acid	1	1	1	1	1	0
Methionine	0	0	0	0	0	0
Arginine	0	0	0	0	0	0
Phenylalanine	0	0	0	0	0	0
Ornithine	0	0	0	0	1	1
Citrulline	0	0	0	0	0	0
BAIB	0	4	4	4	0–1	0–1
Asparagine	0	0	0	0	0	0
Total Units	16	22	25	21	24	22

* Splenectomised patient.
0=none found.

DISCUSSION.

A generalised amino-aciduria has been described in thalassemia by Choremis *et al.* (1957), who detected α-amino-n-butyric acid but no BAIB in the urine of their subjects. Subsequently, Choremis *et al.* (1959) investigated the plasma α-amino-nitrogen levels and urinary amino-acid excretion in thalassemic patients after the administration of protein hydrolysates and concluded that the amino-aciduria was of renal origin. Our results do not confirm the presence of a generalised amino-aciduria, but demonstrate rather excess of one amino-acid—BAIB.

BAIB was shown by Fink *et al.* (1952, 1953, 1956) to be a degradation product of thymine, dihydrothymine and β-ureido-isobutyric acid being intermediary products. Coon (1955) and Kupiecki and Coon (1957) suggested that BAIB could be a product of valine metabolism, but recently Gartner (1959) has shown that thymine is probably the only significant source of urinary BAIB in man.

Patients in whom excessive breakdown of nucleic acids occurs excrete large amounts of BAIB in their urine, as has been demonstrated by Fink (1951a) and Awapara and Schullenberger (1957) in malignancies (and leukaemia) and by Rubini *et al.* (1959) following excessive whole body radiation. BAIB is also excreted in excess in pernicious anaemia (Crane *et al.*, 1958) and March haemoglobinuria (Pare and Sandler, 1954) where disturbances of nucleic acid metabolism are less defined. Increased excretion of BAIB may also occur in generalised amino-aciduria, as in liver necrosis (Walshe, 1953), lead poisoning (Wilson *et al.*, 1953) and scurvy (Jonxis and Huisman, 1954) but in these conditions its significance is probably not as great as when BAIB alone is excreted in excess.

Crumpler *et al.* (1951) and Fink *et al.* (1951b) demonstrated two types of normal individuals – high excretors and low excretors of BAIB. They showed high excretion to be genetically determined in these cases, and further studies by Evered (1956) suggested that the defect was in renal tubular resorption, though the low plasma levels of BAIB make renal clearance studies difficult.

This question of a genetically determined high excretion of BAIB is relevant to the cases of thalassemia here described. The high incidence found in the children examined, in comparison with the low incidence in the 100 normal children previously reported (Hillcoat, in press) and the absence of any high excretors in the families of the patients with thalassemia is evidence against the high excretion of BAIB being genetically determined.

However, the numbers involved are small. These cases are of Mediterranean extraction, but it has not been shown that people of this origin have a higher proportion of excretors than any other group of people.

The excretion of BAIB, therefore, appears to be related to the disease. The question arises whether splenectomy affects this excretion as is suggested by the results, but there has not been an opportunity to investigate this.

Sturgeon and Finch (1957) found that while the turnover of haemoglobin constituents is maximal in thalassemia major, red cell delivery to the peripheral blood is decreased, suggesting that in the marrow of these patients excessive destruction of red cell precursors may occur. The resultant destruction of DNA may be the source of the BAIB.

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A SURVEY FOR ANTIBODIES TO ENCEPHALOMYOCARDITIS VIRUS IN MAN AND ANIMALS

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(*Accepted for publication 1st July, 1960.*)

SUMMARY.

Neutralizing antibodies to the Innisfail strain of EMC virus were present at significant levels, in one of 337 human sera collected in Queensland, and in one of 48 from Papua.

Antibodies were also present, in Queensland, in 22 p.c. of water-rats, *Hydromys chrysogaster*; in 5-10 p.c. of rats, *Rattus rattus, R. norvegicus, R. sordidus conatus* and *Melomys lutillus littoralis*; in 2 p.c. of bandicoots, *Isoodon macrourus*; and in one of 44 horses. A series of mice, cats, dogs and kangaroos examined was negative.

INTRODUCTION.

The presence of a virus of the encephalomyocarditis (EMC) group in Queensland was reported recently (Pope, 1959). Consequently, a serological survey was carried out, in an attempt to determine whether infection of man occurs in Queensland, and to investigate various possible animal reservoirs. In addition, a small series of sera of man and animals from Papua was studied.

METHODS AND MATERIALS.

Stock virus suspensions (Innisfail strain) were prepared from the brains of weaned mice inoculated intraperitoneally and killed when moribund 2-3 days later. Ten p.c. suspensions of brain in 10 p.c. inactivated (60° C. for 30 minutes) rabbit serum saline (RSS) were centrifuged at 2,000 r.p.m. for 10 minutes, ampouled, and stored in a Revco Freezer (— 70° C.). The LD_{50} (calculated by the method of Reed and Muench, 1938) of these stored pools was of the order of $10^{-6.5}$, when tested in weaned mice by the intraperitoneal route. No apparent diminution of titre occurred over periods of up to four months.

For the screen test for neutralizing antibodies, an ampoule of stock virus was thawed in the water-bath at 37° C., and a virus dilution estimated to be at the 100 LD_{50} level was prepared in 10 p.c. RSS. Penicillin and streptomycin were added so that the final dilution in the virus-serum mixture was 100 units/ml. 0·15 ml. aliquots of the virus dilution were then added to equal amounts of unheated, undiluted test sera. A known immune rat serum and a normal rat serum were included as controls in each test. The contents of the ampoule of stock virus used in the test were also titrated in the presence of an equal amount of normal rat serum. The virus-serum mixtures were incubated in a water-bath at 37° C. for one hour, transferred to an ice-bath, and each mixture was inoculated intraperitoneally into a group of six weaned mice (0·03 ml. each). The mice were observed for paralysis and death for 14 days. Survival of four or more mice in a group was accepted as indicat-

ing the presence of neutralizing antibodies in the serum tested, and these sera were further studied to determine their neutralization indices, except in some instances when insufficient serum remained.

Neutralization indices were obtained by the method of addition of serial virus dilutions to constant volumes of undiluted serum. Addition of penicillin and streptomycin, incubation inoculation and period of observation were performed as described in the screen test. Log neutralization indices of 1·7 or greater were considered positive.

The human sera (mainly convalescent) from the Brisbane district were taken mostly from patients with aseptic meningitis, aged between one week and 53 years. Those from the Toowoomba district were from patients in the 3-29 years age range, who were being investigated for evidence of rheumatic fever. Most of the human sera from the Innisfail district were convalescent sera from patients with pyrexia of varied origin, in the age range 6-74 years. The sera collected at Brisbane and Toowoomba were from whites, and 93 out of the 97 from Innisfail were whites (the remainder were aborigines), while those from the Cape York and Gulf of Carpentaria areas, and Popondetta, Papua, were from the native population, and were collected for various survey purposes. All but two of the patients from Cape York and Gulf of Carpentaria were between 4 and 17 years old; one was 4 and the other 35. The Papuans' ages ranged from 18-49 years. Males and females were represented about equally in the series tested from each area, except Innisfail, where the group was predominantly male.

The sera from wild mice were not tested individually, but in pools of up to six mice.

RESULTS AND DISCUSSION.

Five positive results were recorded in the screen tests on 385 human sera, as shown in Table 1. As the convalescent serum of the Innisfail series that was positive in the screen test was exhausted, the acute serum was titrated and showed a log neutralization index of 3·1. This result discounts the possibility that the illness then current was due to EMC virus and, in fact, it was subsequently shown to be leptospirosis. As this man had migrated from Germany to Innisfail in 1943, it is not known where the infection with EMC virus occurred.

The neutralization indices obtained with two of the positive Papuan sera were at equivocal levels (Table 1). There was insufficient of the other two sera to determine their neutralization indices, and in the screen test 5 out of 6 mice

TABLE 1.

Results of neutralization tests on human sera.

Locality	No. of sera	No. positive in screen test	Log neutralization indices
Brisbane	92	0	
Toowoomba	80	0	
Innisfail	97	1	3·1
Cape York and Gulf of Carpentaria	68	0	
Papua	48	4	1·7, 1·2
Total	385	5	

TABLE 2.

Results of neutralization tests on animal sera.

Species	Locality	No. tested	No. positive in screen test	Log neutralization indices
MARSUPIALS				
Isoodon macrourus	Innisfail	50	2	2·0, 2·7
	Brisbane	45	0	
Perameles nasuta	Innisfail	12	0	
Macropus major	Western Queensland	40	0	
Megaleia rufa	Western Queensland	40	0	
RODENTS				
Rattus rattus	Innisfail	36	4	>3, >3, >4·1, 2·7
	Brisbane	58	5	3·1, 3·5, 3·9, 2·6
	Papua	6	1	
R. norvegicus	Innisfail	4	0	
	Brisbane	37	3	1·7, 3·4, 3·6
R. assimilis	Innisfail	9	0	
	Mt. Glorious	15	0	
R. sordidus conatus	Innisfail	32	2	>3
Hydromys chrysogaster	Innisfail	16	5	>3·7, 4·2, 1·8, 3·8, 3·4
	Brisbane	16	2	>4·1, >3·7
	Echuca, Victoria	11	0	
Melomys lutillus littoralis	Innisfail	19	1	2·6
Mus musculus	Innisfail	11	0	
	Brisbane	50	0	
	Toowoomba	54	0	
DOMESTIC ANIMALS				
Cat	Brisbane	30	0	
Dog	Brisbane	61	0	
	Papua	10	0	
Horse	Bundaberg, Townsville, and Mt. Isa districts	44	1	3·2
Total		706	26	

There was insufficient serum of two *R. rattus*, and one *R. s. conatus* to determine neutralization indices.

survived with one serum (infective dose = 39 LD_{50}), and 4 out of 6 survived with the other (infective dose = 199 LD_{50}). In effect, only 1/48 of the Papuan sera can be considered to have possessed neutralizing antibody at a significant level.

The results of the screen tests on animal sera, and the neutralization indices of the positive sera (Table 2) show that rodents are a major reservoir of EMC virus in Queensland. Significant levels of neutralizing antibodies were present in five species, including the introduced rats, *R. rattus* (10 p.c.) and *R. norvegicus* (7 p.c.), and the native rats, *R. s. conatus* (6 p.c.), *H. chrysogaster* (22 p.c.) and *M. l. littoralis* (5 p.c.). The only rodents from which no infections were recorded were 24 allied rats, *R. assimilis*, and 115 house mice, *M. musculus*. Possible explanations of the apparent absence of infection in these two species

are that *R. assimilis* is essentially a rain-forest dweller, and may therefore have less contact with the introduced rats, and that *M. musculus* is highly susceptible to the virus, and so probably would not be an ideal host for maintaining it.

It is interesting that the highest incidence of antibodies in rodents in Queensland (22 p.c. in *H. chrysogaster*) was well below the highest incidence recorded in U.S.A. (87 p.c. in *R. norvegicus* in Mississippi) by Warren *et al.* (1949).

The presence of antibodies in the sera of 4 p.c. of short-nosed bandicoots, *I. macrourus*, from Innisfail suggests that, although rodents constitute the main reservoirs, infection may at times extend to the other small mammals of the same habitat. Previously, Warren *et al.* (1949) had found no evidence of infection in 10 species of small wild mammals other than rats, and it appears, from the results recorded here, that there is little, if any, overflow of infection from rats to cats and dogs in Brisbane.

The presence of a high neutralizing antibody level in a horse from the Bundaberg district was an interesting finding of unknown significance. No survey of the rats in this area was made, but it is quite probable that the virus is present.

It is concluded that EMC virus, although widespread, rarely causes infection of man in Queensland; nevertheless, it remains a potential zoonosis.

Acknowledgments. Human sera, clinical histories, and the majority of the animal sera were collected by Dr. R. L. Doherty and other members of the Institute staff. The water-rats from Victoria were collected by Mr. J. McNally, of the Fisheries and Game Department, Melbourne, and their sera prepared and forwarded by Dr. S. G. Anderson, of the Walter and Eliza Hall Institute of Medical Research, Melbourne.

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